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STUDIES ON THE ENZYMATIC SYNTHESIS OF GLUTAMINE[1,2]

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An enzyme catalyzing the following overall reaction

1. Glutamate + NH_3 + ATP $\overset{Mg^{++}}{\rightleftharpoons}$ Glutamine + ADP + Pi

has been shown to occur generally in plants (7, 8, 25), animals (7, 21) and bacteria (11). If either hydroxylamine or hydrazine is substituted for NH_3, the enzyme forms glutamyl hydroxamate or glutamyl hydrazide at rates about equal to those for glutamine formation. This enzyme was isolated by Elliott in a highly purified form from dried green peas and some of its properties described (8). The possibility that the glutamine synthesizing enzyme may be identical with the enzyme which catalyzes the transfer reactions described by Stumpf and Loomis (22)

2. $GluNH_2 + NH_2OH \longrightarrow GluNHOH + NH_3$

3. $GluNH_2 + N^{15}H_3 \rightleftharpoons GluN^{15}H_2 + NH_3$

has already been considered both by Elliott (8) and by Levintow and Meister (14).

Although the enzyme from green peas has been highly purified by Elliott (8), a number of its important properties have not been reported. Furthermore, the enzyme in its highest state of purity as obtained by the procedure of Elliott is somewhat unstable. We have improved and extended Elliott's purification procedure and obtained an enzyme purified some 4000-fold over the original extract. Data concerning the stability of the enzyme from green peas and its affinity for glutamate, ATP and hydroxylamine have been obtained and are reported here for the first time. Information concerning the specificity of the enzyme and the equilibrium constant of the overall reaction has been determined. These data, when taken with those of Elliott (8), serve to characterize rather completely the nature of the pea enzyme catalyzing the synthesis of glutamine. In addition, further properties of the transfer activities of this system and of the arsenate-activated hydrolysis of glutamine are presented. Finally, data have been obtained by exchange studies with radioactive phosphate and radioactive glutamate which have a bearing on the mechanism of glutamine synthesis and its relation to the transfer activities of the enzyme.

EXPERIMENTAL METHODS

PURIFICATION OF THE ENZYME: The enzyme was extracted from dried green peas, and purified according to the first four steps in the procedure of Elliott (8) (through the first nucleic acid precipitation and extraction). We found the "inhibitor" mentioned by Eliott (8) and partly removed by protamine treatment to be an ATP hydrolase activity and not a true inhibitor. The final steps of the purification procedure reduce this activity to the point where it does not interfere with the glutamine synthesis reaction. The extract from the first nucleic acid fractionation usually contains some (up to 60 % of the total protein) high molecular weight protein which can be removed by centrifugation at 140,000 × g. Removal of this material is essential if one is to obtain a consistent and effective ammonium sulfate fractionation. The fractionation with ammonium sulfate, dialysis and fractionation with nucleic acid were according to the procedure of Elliott (7).

The preparation from the second nucleic acid fractionation (about 1 % protein in 0.05 M phosphate buffer at pH 7.3) was further fractionated by the addition of 0.20 volume of 95 % ethanol. The ethanol was precooled to −20° C and the centrifugation made at −5° C. The inactive precipitate was discarded. The supernatant had a specific activity 4 times that of the starting preparation and represented a 4100-fold increase in specific activity over the original extract. This ethanol fractionation is useful as an additional purification step since it removes inactivated enzyme as well as other proteins. The resulting

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[4] Abbreviations used: Glu, glutamic acid; ATP, adenosinetriphosphate; $GluNH_2$, glutamine; ADP, adenosinediphosphate; Pi, orthophosphate; GluNHOH, glutamylhydroxamate; E, enzyme, TRIS, tris-(hydroxymethyl)-aminomethane; PCMB, *p*-chloromercuribenzoate; Pi^{32}, radioactive orthophosphate; ADP^{32}, radioactive phosphate labeled ADP; ATP^{32}, radioactive phosphate labeled ATP; GSH, glutathione; PPi, inorganic pyrophosphate; AMP, adenosinemonophosphate; As, arsenate; As^{76}, radioactive arsenate.

ethanol solution of the enzyme, although rather dilute, can be kept in solution at −20° C for at least one month without any loss in activity. The enzyme can be precipitated from this solution by increasing the ethanol concentration to 50 %.

Assay: Standard assay conditions for the synthetic activity were as follows: 45 micromoles TRIS buffer (pH 7.8), 50 micromoles potassium glutamate, 2 micromoles ATP, 30 micromoles $MgSO_4$, 10 micromoles β-mercaptoethanol, and 40 micromoles hydroxylamine, per ml of reaction mixture (usually 4 ml). Enough enzyme was added to produce about 0.25 micromole of glutamylhydroxamate per ml in 15-minute incubation at 35° C. The color of the ferric-hydroxamate complex (15) was read on a Klett-Summerson colorimeter with a 540 mμ filter. One unit of activity corresponds to that quantity of enzyme catalyzing the formation of 1 micromole of glutamylhydroxamate per hour under the standard test conditions.

ATP-hydrolase activity was determined by incubating 0.5 ml of enzyme preparation with 0.3 ml of 0.01 M TRIS (pH 7.4) containing 0.001 M $CaCl_2$, and 0.2 ml of 0.01 M ATP for 30 minutes at 25° C (9). The amount of inorganic phosphate enzymatically liberated was then determined.

Standard assay conditions for measurement of transfer activity were as follows: 45 micromoles TRIS buffer (pH 7.4), 30 micromoles $MgSO_4$, 10 micromoles β-mercaptoethanol, 0.5 micromole ADP, 50 micromoles glutamine, 25 micromoles inorganic phosphate, 40 micromoles hydroxylamine per ml of reaction mixture. Enough enzyme was added to produce about 0.25 micromole of glutamyl-hydroxamate per ml in 15 minutes at 35° C.

The hydrolysis of glutamine in the presence of arsenate and phosphate was followed by chromatographing a 3 λ spot of each reaction mixture with 88 % phenol, developing the color with ninhydrin and reading the maximum intensity of each spot according to the method of Block (3). Absolute values were determined by running a series of glutamine and glutamic acid standards under the same conditions.

Phosphate labeled ADP was prepared by incubation of P_i^{32} and ATP with fresh sheep heart homogenate according to the method of Hems and Bartley (10). Labeled ADP and ATP were separated from the incubation mixture by paper chromatography according to Bandurski and Axelrod (2). The labeled phosphate compounds resulting from the exchange experiments were also separated by paper chromatography, the spots located with molybdate reagent, cut out, and counted with a 1.4 mg/cm^2 end window tube and a standard scaling circuit.

Results

The Synthesis Reaction, *Stability of Enzyme:* The stability of the enzyme is indicated by the maintenance of full activity of dilute ethanolic solutions of the enzyme for periods up to one month at −20° C. As a matter of practical interest in the consideration of possible purification procedures, the pH stability of the enzyme was determined (fig. 1). At room temperature it is markedly unstable particularly below pH 6, in periods as short as one hour.

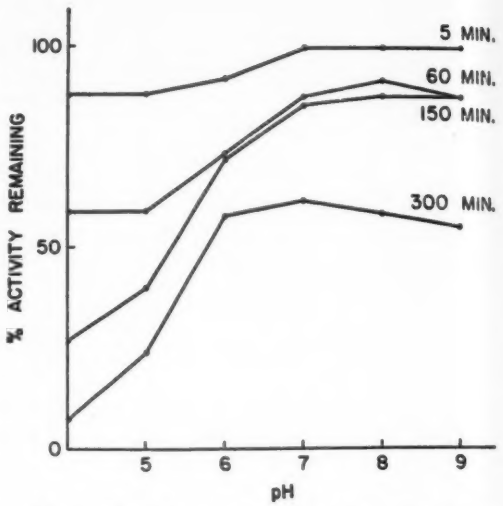

Fig. 1. The effect of pH on the stability of the glutamine synthesizing enzyme. Each sample was preincubated at 25° C at the pH values shown.

Equilibrium: The equilibrium constant of the synthesis reaction was determined by approaching the equilibrium from the glutamine side and measuring the net synthesis of ATP from ADP and radioactive inorganic phosphate. Figure 2 shows the data obtained in one of these experiments. The equilibrium constant calculated for the forward reaction at pH 7.4 is 1.7×10^3. It can be calculated from the equilibrium

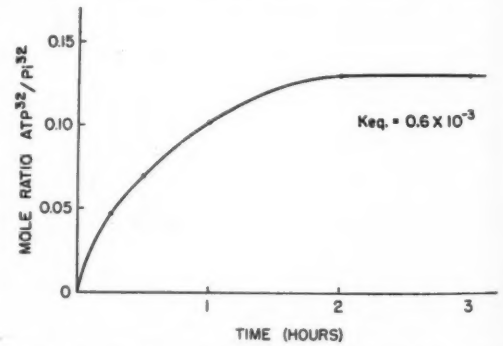

Fig. 2. Evidence for the reversibility of the glutamine synthesis reaction. Each sample contained: 45 micromoles TRIS buffer (pH 7.4), 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 2 micromoles ADP, 50 micromoles glutamine, 5 micromoles of P_i^{32}, 27 units of enzyme/ml of reaction mixture. Temperature of incubation, 35° C. Synthesis of ATP is expressed as mole ratio of ATP formed to orthophosphate conc at the time ATP conc is measured.

value that the free energy of formation of the γ-glutamylamide bond is about 4,800 calories less than for the pyrophosphate of ATP. This surprisingly small difference may indicate that the amide bond of glutamine has a higher free energy of formation (3,500 cal) than the amide bond of asparagine (4), or it may indicate that the commonly accepted value of 10,500 cal for the ΔF of the formation of the terminal phosphate linkage of ATP is too high. Levintow and Meister (14) have recently determined this same equilibrium constant by a different method and arrived at a value of 1.2×10^3 at pH 7.0 and 37° C. They make the assumption that the ΔF of formation of the amide bond of glutamine is the same as that for asparagine and suggest that the true ΔF of formation of ATP from ADP and Pi is near 8,000 cal. An independent confirmation of this comes from the calculations of Anfinsen and Kielley (1).

Properties of the Enzyme: Although Elliott (7) has determined the reactant affinities of the enzyme from sheep brain, the same data have not previously been reported for the enzyme from peas. The initial rates of the synthetic reaction as a function of the concentration of glutamate, ATP, and hydroxylamine are shown in figure 3. The relatively high affinity of the enzyme for hydroxylamine suggests the usefulness of this system as a trapping system for low concentrations of hydroxylamine. It is noteworthy that potassium could not be shown to be required for the synthesis of glutamine. This is in contrast to the potassium requirement for the synthesis of glutathione (19) and of γ-glutamylcysteine (26).

Although iodoacetate and oxidized glutathione in concentrations up to 1×10^{-2} M and an excess of solid

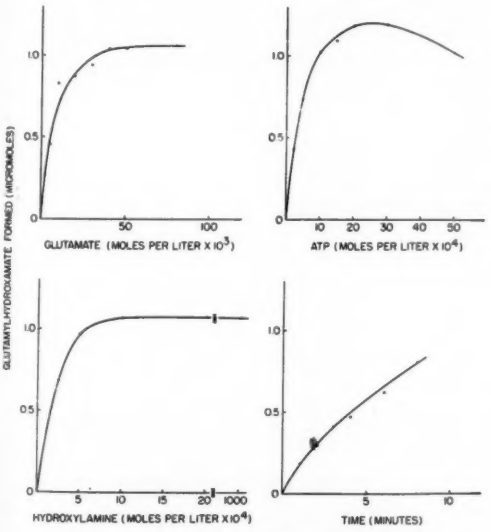
GLUTAMYLHYDROXAMATE FORMED (MICROMOLES)
GLUTAMATE (MOLES PER LITER X 10³)
ATP (MOLES PER LITER X 10⁴)
HYDROXYLAMINE (MOLES PER LITER X 10⁴)
TIME (MINUTES)

FIG. 3. The rate of the synthesis reaction as a function of the concentration of reactants, and as a function of time. Assay conditions were as already described with the exception of the variable being studied.

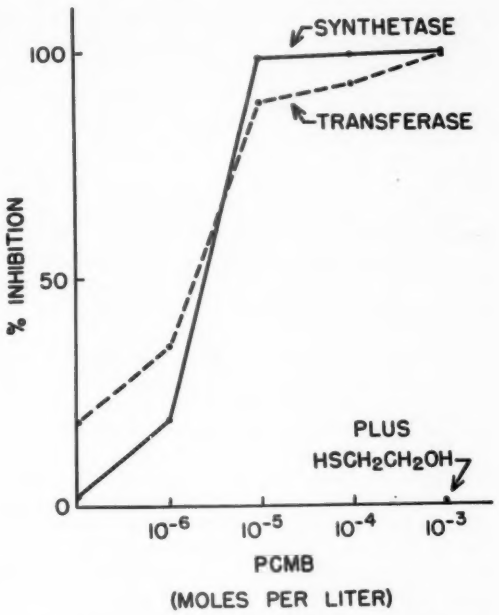

FIG. 4. Para-chloromercuribenzoate as an inhibitor of the transfer and synthesis reactions. Experimental conditions were identical to the standard assay procedures, with the exception that β-mercaptoethanol was omitted except where noted.

cystine all fail to inhibit the synthetic activity of the enzyme, 1×10^{-3} M PCMB completely inhibits it (8). Figure 4 shows the dependence of the inhibition of glutamine synthetase on PCMB concentration. This inhibition is completely reversed by β-mercaptoethanol. These facts indicate the participation of at least one sulfhydryl group during the reaction and demonstrate that this group (or groups) is not readily available for reaction with all sulfhydryl reagents.

Table I presents additional data demonstrating the inhibitory effects of the end products ADP and Pi.

TABLE I

EFFECT OF ADP, PHOSPHATE, ARSENATE AND ARSENITE ON THE RATE OF GLUTAMYLHYDROXAMATE SYNTHESIS

SYSTEM	GLUNHOH	INHIBITION
	micromoles	%
Complete	1.00	..
+ ADP (1 micromole/ml)	0.75	25
+ Phosphate (12 micromoles/ml)	0.55	45
+ ADP and phosphate	0.41	59
+ Arsenate (12 micromoles/ml)	1.22	22 % stimulation
+ Arsenite (12 micromoles/ml)	0.92	8

The complete system components were the same as those described for the standard assay.

The arsenate effect on the synthetic reaction is especially interesting as arsenate has already been shown (23) to enhance markedly glutamyl transfer activity.

The purified enzyme would not catalyze the formation of hydroxamates of any of the folowing amino acids: aspartic acid, glycine, alanine, β-alanine, arginine, valine, leucine, cysteine or lysine. Aspartic acid and glycine inhibited the formation of glutamylhydroxamate. A concentration of glycine or aspartate nine times that of the glutamate, inhibited glutamylhydroxamate formation by 50 %. The enzyme does catalyze the formation of D- and L-α-amino-adipylhydroxamates at a rate of about 1/20 that for the formation of glutamylhydroxamate (12).

In a preliminary report (30) we suggested a reaction mechanism for the enzymatic synthesis of glutamine which involved (a) phosphorylation of the enzyme by ATP as a first step, (b) reaction of the enzyme-phosphate with glutamate to form an enzyme-glutamate complex, and finally (c) reaction of the enzyme-glutamate complex with ammonia or hydroxylamine to form the amide or hydroxamate. It was also suggested that the transfer reaction involved formation of an enzyme-glutamate intermediate from glutamine and enzyme, followed by reaction of the intermediate with ammonia or hydroxylamine.

In light of the fact that the transfer reaction (reaction c above) requires ADP and inorganic phosphate or arsenate (as well as magnesium or manganous ions) (8), one might expect reaction c to be rate-limiting in the overall synthetic reaction at the start of the reaction when there is no ADP or inorganic phosphate present. If this were true, the synthetic reaction in the initial stages should have the kinetics of an autocatalytic reaction. This is, however, not the case, as the data presented in figure 3 show. These facts suggest that the mechanism postulated earlier needs further elaboration, even though it does satisfactorily account for the fact that intermediates such as glutamylphosphate have not been found. That the mechanism proposed is incomplete is evidenced too, by the fact that it does not account for the ADP, Pi or arsenate requirement for the transfer reaction. This reaction has, therefore, been studied in detail.

THE TRANSFER REACTION: Enzyme systems which catalyze the exchange of the amide group of L-glutamine for either ammonia or hydroxylamine (reactions 2 and 3) have been shown to have a widespread occurrence in plants (6, 8, 22, 23), animals and bacteria (11, 24). The systems extracted from plant and animal tissues appear to have identical cofactor requirements, these being manganous or magnesium ions, ADP or ATP and phosphate or arsenate. Bacterial extracts may contain at least two different systems which catalyze reactions 2 and 3. One system apparently has no cofactor requirement although its activity is enhanced by cupric ions (24). The second system requires manganous ions, phosphate or arsenate and possibly ADP (24).

The ratio of the synthetic activity to the transfer

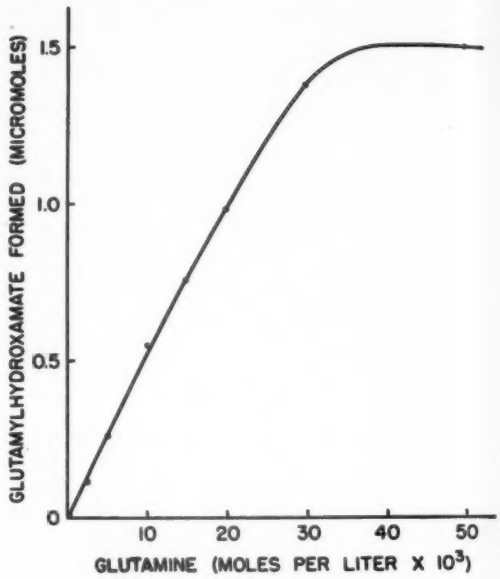

FIG. 5. Rate of the transfer reaction as a function of glutamine conc. Experimental conditions identical to the standard assay procedure, with the exception of the glutamine conc.

activity of the enzyme preparations was checked at each stage in the purification procedure and, in confirmation of Elliott's results (8) was found to be constant throughout the purification. As with the synthetic activity, 1×10^{-2} M iodoacetate and 1×10^{-2} M oxidized glutathione were ineffective as inhibitors. However, *p*-chloromercuribenzoate was as effective as an inhibitor of the transfer reaction as of the syn-

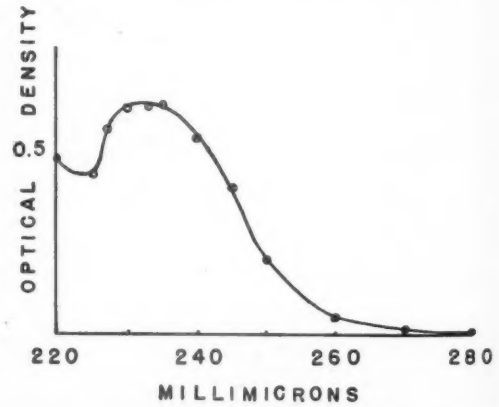

FIG. 6. Difference spectrum indicating complex formation between glutamine synthetase and PCMB. One cuvette contained 0.1 mg purified enzyme in 3.10 ml, the second cuvette contained 0.1 mg purified enzyme and 100 micromoles of PCMB, the third cuvette (blank) contained 100 micromoles of PCMB.

thetic reaction. β-Mercaptoethanol completely reversed both inhibitions. Figure 4 compares the inhibition of the two reactions as a function of the concentration of PCMB. The differences in the degree of inhibition are small, but real. The general similarity of the inhibition curves suggests that the same sulfhydryl group (or groups) may be involved in the two catalytic functions. We have already suggested (30) that some enzyme-bound intermediate may be common to the two reactions.

With the thought in mind that one of the enzyme-bound intermediates in the synthesis reaction and transfer reaction might be an enzyme-thiol ester, an attempt was made to find optical evidence (9) for such a linkage. Using a Beckman DU spectrophotometer with the Beckman photomultiplier attachment, no evidence for such a linkage could be found although a peak at 235 mμ for the complex formation of the enzyme with PCMB (5) was readily observable (fig 6).

The small difference in the degree of inhibition of the two activities may somehow result from the fact that in one case the enzyme-bound intermediate is formed by the approach of glutamate to the enzymatic site and in the other by the approach of glutamine (L-glutamine obtained from Nutritional Biochemicals Corporation). In connection with the fact that the syntheic reaction is less sensitive than the transfer reaction to PCMB at the lower concentrations, it is noteworthy that the affinity of glutamate for the enzyme (fig 2) is greater than that of glutamine (fig 5).

The relative effectiveness of ADP and ATP as cofactors from the transfer reaction is shown in figure 7. The results confirm those of Stumpf (23) who used a transfer system from pumpkin seedlings. Even though ATP preparations are effective it is by no means certain that it is ATP itself which has the activity (16). An ATP preparation containing about 6 % ADP would give the results shown in figure 7 if one assumes that ATP does not compete effectively with ADP for some two point attachment necessary to the activity, but is effective in a one point attachment which causes inhibition. Since the enzyme preparation itself has an ATP-hydrolase activity it would be very difficult to prevent the formation of ADP during a reaction in which ATP was added as a cofactor. However, an experiment designed to detect an increase in rate of the transfer reaction with time in the presence of ATP as a cofactor showed the rate to be constant from zero time, i.e., the rate does not increase as more ADP is liberated from ATP. AMP is ineffective as a cofactor in this reaction.

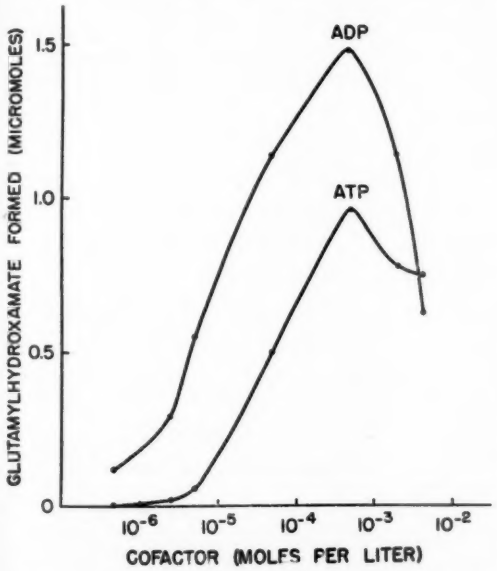

FIG. 7. ADP and ATP as cofactors in the transfer reaction. Experimental conditions were according to the standard assay procedure except for the variable being studied.

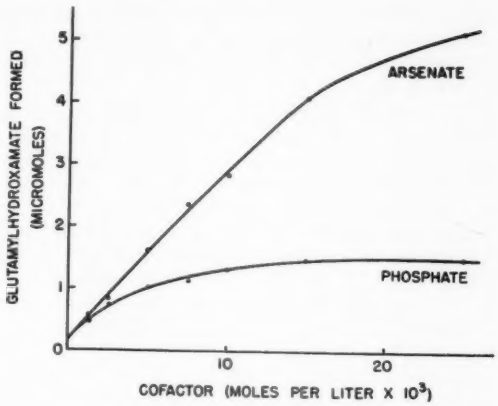

FIG. 8. Phosphate and arsenate as cofactors in the transfer reaction. Conditions were according to the standard assay procedure except for the variable being studied.

That arsenate is more effective than phosphate as a cofactor in the transfer reaction has been already noted by Stumpf et al (23). Figure 8 shows that this greater effectiveness holds true only at relatively high concentrations of phosphate and arsenate. Since the difference in the effectiveness of the two cofactors is very small at low concentration, it seems reasonable to suggest that the differences in the maximum potential effectiveness may also not be very great. In the case of phosphate this maximum is never realized because a high concentration of phosphate tends to drive the reaction toward the formation of glutamate, ATP and ammonia, possibly binding a part of the enzyme in the form of some phosphate-containing intermediate more stable than the one which reacts with hydroxylamine to form glutamylhydroxamate. The corresponding arsenate complex might be expected to be less stable, resulting in a higher concentration of enzyme available to carry out the transfer reaction

and, therefore, in an apparently greater effectiveness of arsenate as a cofactor. Arsenite is not effective as a cofactor in the transfer reaction but is only slightly inhibitory.

THE ARSENATE-ACTIVATED HYDROLYSIS: In addition to the transfer and the glutamine synthesis reactions, there is a third reaction catalyzed by this same enzyme preparation from peas. This is an arsenate-activated hydrolysis of glutamine (13, 14, 23). The reaction has an absolute requirement for ADP and magnesium or manganous ions (14) as well as for arsenate ions. It can be seen from the data in table II that the rate of the reaction is linear with time through the hydrolysis of 19 % of the glutamine present. ATP strongly inhibits the reaction. It is of interest to compare the production of glutamate when phosphate is substituted for arsenate. There is, of course, a little glutamate formed due to reversal of the synthesis reaction. When hexokinase and glucose

TABLE II

ARSENATE-ACTIVATED HYDROLYSIS OF GLUTAMINE BY GLUTAMINE SYNTHETASE FROM PEAS

CONDITIONS	TIME	GLUTAMATE FORMED
	min	*micromoles/ml*
Complete with arsenate	15	1.0
Complete with arsenate	30	2.2
Complete with arsenate	60	4.7
+ATP (0.5 micromoles/ml)	60	2.2
Complete with phosphate	60	0.02
+hexokinase and glucose ..	60	0.9

The complete system contained: 45 micromoles TRIS (pH 7.4), 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 1 micromole ADP, 25 micromoles arsenate or phosphate, 25 micromoles glutamine, and 70 units of enzyme per ml. Total reaction mixture was 4.0 ml. One mg of hexokinase (Pabst) and 7.2 mg of glucose were added where indicated.

are added to trap out the ATP as it is synthesized, there is a fairly rapid "hydrolysis" of glutamine. The rate of the reaction under these circumstances, however, is still only about 20 % of the rate of the arsenate-activated reaction. Because of the difference in rates of the arsenate system and the phosphate-hexokinase-glucose system, it appears that the step which is rate-limiting in the transfer reaction is also limiting in the hydrolysis reaction. It must be then that the arsenate-activated reaction proceeds rapidly because of the instability of some organic arsenate compound (or complex) formed during the reaction and which decomposes more rapidly than phosphate is transferred to ADP.

The degree of specificity of this enzyme with respect to the compounds which it activates for the transfer reaction has been noted by Stumpf (23) and by Levintow and Meister (14). The formation of glutamine from glutamylhydroxamate has not been detected even in the presence of a large excess of ammonium ions, indicating that the activation of glutamyl hydroxamate proceeds at a slow rate, as compared to glutamine activation.

From the results presented in table V, it appears that glutamate remains bound to the enzyme during the transfer reaction. The relation of this fact to mechanism proposals will be considered in the discussion section of this paper.

EXCHANGE REACTIONS: When the highly purified enzyme was used in exchange experiments similar to those reported earlier (30), it was found that there was essentially no exchange of Pi^{32} into ATP in the presence of glutamate unless a catalytic quantity of ammonia was simultaneously present.

Figure 9 shows the dependence of the exchange on the concentration of ammonium ions. That the exchange is not completely dependent upon the addition of ammonia is probably due to the presence of a small quantity of ammonia adsorbed on the enzyme. The earlier results (30) in which there was no apparent requirement for ammonia are probably due to the fact that the 270-fold purified enzyme still contained traces of ammonia. Also it is likely that the exchange experiments reported by Levintow and Meister (14), in which exchange of Pi^{32} into ATP was not depend-

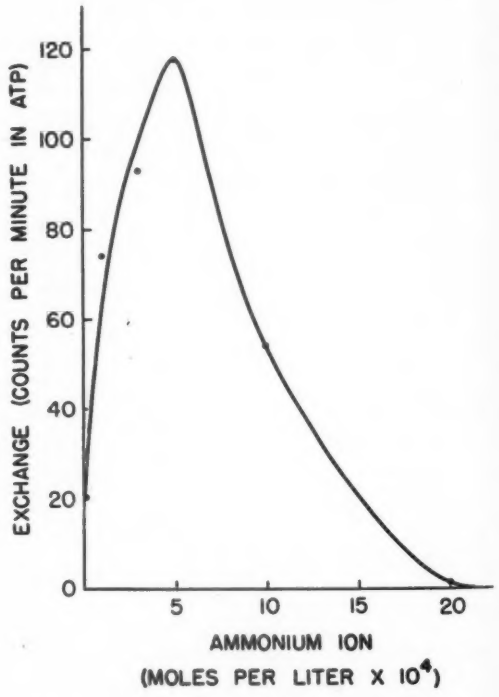

FIG. 9. The exchange of P^{32} into ATP as a function of ammonia conc. The complete system contained: 2.5 micromoles Pi^{32}, 2 micromoles ATP, 50 micromoles glutamate, 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 45 micromoles TRIS buffer (pH 7.4) and 40 units of enzyme/ml of reaction mixture. The incubation was at 35° C for 1 hr.

TABLE III

EXCHANGE OF Pi^{32} INTO ATP, CATALYZED BY PURIFIED GLUTAMINE SYNTHESIS

CONDITIONS	ATP	Pi
	cpm/10	*cpm/10*
Complete system	200	3600
– Ammonia	28	3800
– Ammonia + hydroxylamine	31	3800
Complete + PCMB to 10^{-6} M	170	3600
Complete + PCMB to 10^{-5} M	9	3800
Complete + PCMB to 10^{-5} M + β-mercaptoethanol	200	3600

The complete system contained: 2.5 micromoles Pi^{32} (380,000 cpm), 2 micromoles ATP, 50 micromoles glutamate, 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 45 micromoles TRIS buffer (pH 7.4), 12 micromoles ammonia (or hydroxylamine), 40 units of enzyme /ml of reaction mixture. Incubation was at 35° C for 1 hr.

ent upon added ammonia, can be explained on the basis of trace quantities of ammonia in their enzyme preparation. The measured exchange of Pi^{32} into ATP decreases to near zero at the higher concentrations of ammonia where all of the ATP initially present is used up.

The exchange of Pi into ATP, mediated by highly purified glutamine synthetase, did not occur unless all of the reactants and cofactors necessary for the overall synthesis reaction were present. Nor did exchange occur with catalytic quantities of hydroxylamine in place of ammonia. This is in agreement with the observation mentioned earlier that the rate of the transfer of the glutamyl radical from hydroxylamine to ammonia is very slow.

The data recorded in table III demonstrate that this exchange reaction is inhibited by the same levels of PCMB which are effective in inhibiting the synthetic reaction and the transfer reaction. Inhibition of the phosphate exchange reaction is also completely reversed by the addition of β-mercaptoethanol.

As illustrated by the data of table IV, exchange of ADP^{32} into ATP is also dependent upon the simultaneous presence of the full complement of reagents and cofactors necessary for the overall synthetic reaction. The rate of exchange of ADP^{32} into ATP equalled the rate of exchange of Pi^{32} into ATP.

TABLE IV

EXCHANGE OF ADP^{32} INTO ATP CATALYZED BY PURIFIED GLUTAMINE SYNTHETASE

CONDITIONS	ADP	ATP
	cpm/200	*cpm/200*
Complete system	174	226
– Ammonia	390	12
– Glutamate	400	7
– Ammonia and glutamate	400	15
– Enzyme	400	0

The complete system contained: 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 45 micromoles TRIS buffer (pH 7.4), 0.5 micromoles ADP^{32}, 2 micromoles ATP, 1.2 mocromoles ammonia, 50 micromoles glutamate, 2.5 micromoles Pi, and 40 units of enzyme per ml of reaction mixture. Incubation was at 35° C for 2 hrs.

In an exchange experiment in which D-glutamate replaced L-glutamate, the ratio of Pi^{32} exchanged into ATP in the presence of D-glutamate to that exchanged into ATP in the presence of L-glutamate was 10/280. Levintow and Meister (12) have reported that although the enzymatic synthesis of D-glutamine proceeds at about 40 % of the rate of the synthesis of L-glutamine, the rate of transfer of the glutamyl group from D-glutamine to hydroxylamine is only 3.93/286 of the rate of that transfer from L-glutamine. This similarity between the D-glutamate/L-glutamate ratios of the rates of the exchange reactions and the transfer reactions is further evidence that there can be no exchange of Pi^{32} or ADP^{32} into ATP except by the reversal of the overall synthetic reaction. The optical specificity of the enzyme which permits only a slow rate of formation of the hydroxamate from D-glutamine, also prevents a rapid exchange of Pi^{32} into ATP.

TABLE V

INCORPORATION OF GLUTAMATE-C-14 INTO GLUTAMINE DURING THE TRANSFER REACTION

SAMPLE	$GluNH_2$	Glu	GLUTAMATE EXCHANGED	GLUTAMATE TRANSFERRED
	cpm/ml	*cpm/ml*	*micromoles*	*micromoles*
Complete with phosphate	5,600	318,000	0.13	6.5
Complete with arsenate	2,000	340,000	0.04	9.0

The complete reaction mixture contained 30 micromoles Mg^{++}, 45 micromoles TRIS, 10 micromoles β-mercaptoethanol, 0.5 micromole ADP, 40 micromoles NH_4^+, 25 micromoles L-$GluNH_2$, 5 micromoles D, L-Glu-C^{14}, 5 micromoles L-Glu carrier and 12.5 micromoles Pi or As as indicated/ml of reaction mixture. Enough enzyme was added to each ml of reaction mixture to cause the formation of 6.5 micromoles of GluNHOH in the presence of Pi and 9.0 micromoles of GluNHOH in the presence of As, during the incubation period, when NH_2OH was substituted for NH_4^+.

Figure 10 shows the effect of arsenate on the apparent equilibrium of the glutamine synthesis reaction (approached from the glutamine side) as measured by incorporation of Pi^{32} into ATP. The possibility that the decrease of ATP^{32}/Pi^{32} in the presence of arsenate was due to the formation of an ADP-arsenate compound with an appreciable stability was considered and tested by incubating glutamine, ADP, magnesium and radioactive arsenate (As^{76}) with the enzyme. The reaction was stopped by cooling to 0° C, and an aliquot of the reaction mixture chromatographed at 0° C using the procedure of Bandurski and Axelrod (2). The chromatography required 4 hours, during which time the As^{76} spots, containing

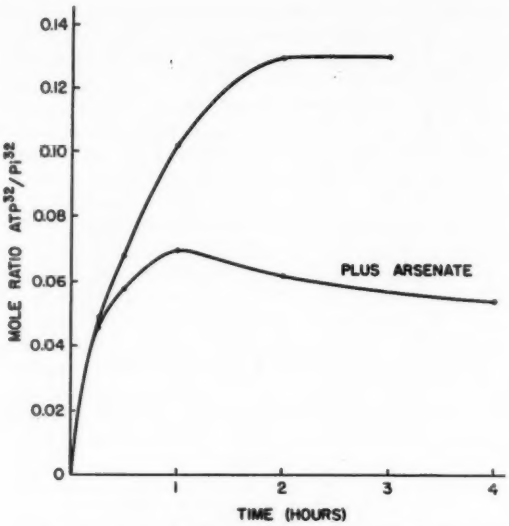

FIG. 10. The effect of arsenate on the equilibrium for the synthesis of glutamine. The incubation mixture contained: 45 micromoles TRIS buffer (pH 7.4), 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 2 micromoles ADP, 50 micromoles glutamine, 5 micromoles of Pi^{32}, and 27 units of enzyme/ml of reaction mixture.

30,000 counts, moved about 5 inches. No radioactive spot other than that of inorganic arsenate could be detected and the arsenate spots were perfectly symmetrical with no indication of tailing which would suggest the presence of an arsenate ester which decomposed during the chromatographic development. From these data we have estimated that if ADP-arsenate is produced under the present conditions, it must have a half-life of less than 15 minutes. The fact that the ratio of ATP^{32}/Pi^{32} at equilibrium is much lower in the presence of arsenate is, therefore, a result of the arsenate-activated hydrolysis of glutamine, by which glutamate and ammonia are formed and the apparent equilibrium point shifted from that obtained in the absence of arsenate. The decrease of the ATP^{32}/Pi^{32} ratio with time (after 1 hour) is a further measure of this hydrolysis.

DISCUSSION

The data presented here, together with the results of other investigators, serve to characterize three primary catalytic activities of the glutamine synthesizing system and strongly suggest that these three activities are the manifestation of the properties of a single protein. This characterization is of importance because of the contribution which it makes to our appreciation of the complexity of the mechanism of the synthetic reaction. The manner in which this reaction proceeds has been the subject of speculation and experiment from the time of the earliest reports of Speck on the synthesis of glutamine. By analogy with other biosyntheses involving ATP, the reaction was first suggested to involve the formation of a γ-glutamyl phosphate intermediate which would then react with ammonia (perhaps non-enzymatically) to form glutamine. Vigorous efforts by several investigators failed, however, to show any direct evidence for such an intermediate.

The exchange experiments reported here indicate that the only intermediates formed during the synthesis of glutamine are enzyme-bound intermediates. It would appear that further exchange experiments with Pi^{32} or ADP^{32} would not be capable of yielding further information about possible reaction mechanisms.

In an attempt to account for the ADP and phosphate requirements of the transfer reaction, Levintow and Meister (14) have suggested that the transfer reaction consists of a complete reversal of the synthetic reaction followed by the synthetic reaction. Consideration of the following evidence renders this theory unlikely. The extremely low concentration of ATP formed as a result of the reverse reaction would permit the forward reaction to proceed at a rate slow in comparison to the transfer rate actually observed. One could conclude from their theory that the several-fold greater effectiveness of arsenate as a cofactor as compared to phosphate, in the transfer reaction is the result of the formation of the ADP-arsenate analog of ATP and that this ADP-arsenate compound is a more effective reagent in the synthesis reaction than ATP. Data already presented indicate that either ADP-arsenate is not formed, or, if formed, has a very short half-life. In either event the data do not permit the conclusion that ADP-arsenate is an intermediate in the glutamine transfer reaction. Furthermore, the proposal that the transfer reaction occurs by a reversal of glutamine synthesis followed by a resynthesis of glutamine, is inconsistent with the facts that manganous ions are more effective in the transfer reaction while magnesium ions are more effective in the synthetic reaction. Finally, if this mechanism were the correct one, there should be an exchange of radioactive glutamate into glutamine during the transfer reaction, the rate of this exchange should equal the rate of the transfer reaction and it should be increased by the addition of arsenate. That such is not the case has been shown in table V. The rate of exchange is about 2 % of the rate of transfer and this rate of exchange is decreased by the addition of arsenate in the place of phosphate, whereas the rate of the transfer reaction is increased by arsenate.

The data presented in this paper suggest the following conclusions concerning the mechanisms of the reactions catalyzed by the glutamine synthesizing enzyme: 1) All intermediates are enzyme-bound. 2) The formation of a simple enzyme-phosphate, enzyme-pyrophosphate, enzyme-ADP, or enzyme adenylate complex can no longer be considered as likely first steps in the synthetic reaction. More complicated intermediates must, therefore, be sought.

There is as yet no direct unequivocal evidence for

such an intermediate. All of the published data are, however, consistent with the hypothesis that glutamate, ATP and enzyme interact to form a glutamate-ATP-enzyme complex which can then react reversibly with ammonia to form glutamine, ADP and Pi. The formation of this complex from the glutamate side would appear to be more rapid with magnesium ions as compared to manganous ions, while its formation from the glutamine side is more rapid in the presence of manganous ions. It would seem that if the complex exists, it must be quite stable since the exchange of Pi^{32} and ADP^{32} into ADP is very slow in the absence of ammonia. The corresponding arsenate complex is evidently more reactive, thus causing a more rapid transfer reaction as well as a more rapid hydrolysis of glutamine.

It is interesting to compare and contrast these suggestions concerning the mechanism of the synthesis of the amide bond of glutamine with the recently proposed mechanisms for the ATP-dependent syntheses of certain peptide bonds (17, 18, 20, 28, 29).

The evidence presented by these workers suggests phosphorylation of the enzyme as an initial step in the enzymatic formation of these particular peptide bonds. However, since extremely low concentrations of ammonia are so effective in causing exchange of Pi into ATP in the glutamate synthesizing system, perhaps a re-examination of these other systems, using carefully purified enzymes, would be worthwhile. It appears possible, however, that the reaction mechanism for the formation of the amide bond of glutamine may be fundamentally different from those reaction mechanisms presently thought to be involved in peptide bond synthesis.

Summary

An improved method has been described for obtaining from dried green peas in highly purified form the enzyme catalyzing the synthesis of glutamine from glutamate and ammonia. The equilibrium constant for the overall reaction has been determined and the enzyme has been characterized with respect to its affinities for the reactants. The glutamyl transfer reactions of this enzyme have been further characterized. It is concluded that these transfer reactions and the arsenate-activated hydrolysis of glutamine are manifestations of the overall synthetic activity of the enzyme. The mechanism of the synthesis reaction has been studied by means of the enzyme-catalyzed exchange of radioactive orthophosphate and ADP into ATP. The existence of a glutamate-ATP-enzyme complex as an intermediate is indicated and it is suggested that this intermediate is common to the glutamine synthesis reaction and the glutamyl transfer reactions.

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SUCCINOXIDASE AND CYTOCHROME OXIDASE IN BARLEY ROOTS[1,2]

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It has been suggested that the terminal oxidase concerned with the normal respiration in most plants is cytochrome oxidase (7, 8). Evidence from reconstructed systems suggests that other enzymes can also operate as terminal oxidase systems (2, 15, 21, 23, 25, 28, 30). Although tyrosinase and ascorbic oxidase may function there is no experimental evidence that they make energy available for endergonic processes. There is evidence to associate salt accumulation and salt respiration with the cytochrome system (19, 29). If no cytochrome oxidase were present in tissues which accumulate salt some revision of the Lundegårdh hypothesis (20, 24) of salt accumulation would be required. Consequently, it is especially interesting that James presents evidence that ascorbic oxidase is a terminal oxidase of barley root tips (10, 12) and implies that this oxidase is concerned in salt accumulation (11). James has been unable to extract cytochrome oxidase or detect cytochrome absorption bands in barley roots more than 5 to 6 days old.

The direct demonstration of cytochrome oxidase in older wheat roots has also failed (4, 28), although the characteristic absorption bands of the cytochromes have been observed in the intact roots (20); thus suitable procedures for extraction and assay may be difficult to find.

In barley as in wheat roots the negative results (10, 12) might mean only that cytochrome oxidase is difficult to extract. Since it has been generally accepted that oxidation of succinate proceeds through the classical cytochrome system (8), the demonstration of succinate oxidation and formation in barley roots (16, 17) suggests that succinoxidase and, thus, cytochrome oxidase function. Therefore, an attempt was made to isolate cytochrome oxidase and, as a confirmatory test, the succinoxidase system.

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[2] This is a report of research completed during the tenure of a U. S. Educational Exchange Grant, authorised under Public Law 584, 79th Congress, the Fulbright Act.

Methods and Materials

Preparation of Barley: Cape barley (*Hordeum vulgare*) was used as the source of roots. The grains were soaked in distilled water for 3 to 7 hours at 25° C with 2 or 3 changes of water. In some experiments, an initial soak of one hour in 0.6 % H_2O_2 was used to reduce fungal contamination. The soaked grains were allowed to germinate on wet filter paper in a saturated atmosphere and the seedlings were held thus at 25° C for 2 days. For experiments in which longer growth periods were required, seedlings were transferred after 3 days to waxed boards, with holes through which the roots grew in aerated half-saturated $CaSO_4$ solutions for periods up to 7 days in a room at 25° C. After this treatment the roots were profusely covered with root hairs and often had a curled appearance. The maximum amount of root material that could be grown conveniently for an experiment was about 10 grams.

Preparation of the Enzyme Extract: The root system of the 2-day-old seedlings was excised at the seed and that of 10-day-old seedlings at about 5 cm from the seed. In some experiments the apical cm was removed but no differences between the root segments were found. After being thoroughly rinsed with distilled water the roots were chilled to 5 to 7° C. The following operations were carried out in a cold room at 0 to 5° C. The roots were washed in the homogenizing medium at 2° C and cut into about 0.5-cm segments. The segments were blotted to remove excess fluid and homogenized in fresh medium, 1.5 gm of roots to 5 ml of medium. Maceration was carried out by hand with a conical Pyrex glass homogenizer, consisting of an outer tube which was immersed in a water-ice bath and a hollow pestle which contained a water-ice mixture. Cooling was important since homogenization of roots required a vigorous action. Cell debris from the unstrained homogenate was removed by centrifugation for 10 minutes at $1000 \times g$. The resulting supernatant was centrifuged at about $10{,}000 \times g$ for 25 to 30 minutes at -2 to 0° C

in a refrigerated centrifuge. The residue thus obtained was resuspended in the homogenizing medium and again sedimented at about 10,000 × g for 25 to 30 minutes. After the supernatant was decanted, the excess liquid in the centrifuge tube was removed with filter paper, the residue was weighed and resuspended with the homogenizing medium. The residue weights averaged about 3 % of the fresh root weights for 2-day-old seedlings and 2 % for the 10-day-old seedlings.

HOMOGENIZING AND ASSAY MEDIUM: Various homogenizing and assay media were tested. The final media which consistently allowed the demonstration of succinoxidase activity contained the reagents listed in table I. The homogenizing medium was hypertonic to the barley roots.

Preparations without cysteine in the homogenizing medium did not show endogenous oxygen uptake or succinoxidase activity although cytochrome oxidase was demonstrated. Variation of the cysteine concentration over the range 10^{-4} to 10^{-2} M was not found to influence the succinoxidase activity. Cysteine was not required in the assay medium.

The presence of EDTA [3] was especially effective in decreasing "autoxidation" of ascorbate. Presumably EDTA chelated contaminating metal ions. The routine addition of crystalline insulin as a source of pure protein presumably also served to bind metal ions. No consistent effect in stabilizing enzymatic activity was noted although some reduction of ascorbate autoxidation was found. The low solubility of insulin, however, may have prevented the introduction of effective amounts. Provided that EDTA were present, the autoxidation of ascorbic acid was low so no corrections were applied. Ascorbic acid to be added was usually neutralized by TRIS rather than by an inorganic base in order to reduce non-enzymatic oxidation of ascorbate.

TABLE I

COMPOSITION OF THE HOMOGENIZING MEDIUM AND ASSAY MEDIA FOR SUCCINOXIDASE AND CYTOCHROME OXIDASE TO SHOW CONSISTENT ACTIVITY

COMPONENT	INITIAL CONCENTRATIONS		
	HOMOGENIZATION (M)	SUCCINOXIDASE ASSAY (M)	CYT OXIDASE ASSAY (M)
Cysteine hydrochloride	5.00×10^{-3}	1.25×10^{-3}	1.11×10^{-3}
Insulin	50.0 mg/l	12.5 mg/l	11.1 mg/l
EDTA	1.00×10^{-2}	2.50×10^{-3}	2.22×10^{-3}
Phosphate buffer, pH 7.5	1.00×10^{-1}	1.00×10^{-1}	8.90×10^{-2}
K-succ, pH 7	....	2.00×10^{-2}	1.78×10^{-2}
Cyt c	....	6.40×10^{-5}	5.70×10^{-5}
TRIS	....	....	4.40×10^{-2}
Ascorbic acid	....	....	3.95×10^{-2}
Sucrose	1.80×10^{-1}	1.43 to 2.40×10^{-1}	1.27 to 2.13×10^{-1}
Calculated tonicity	0.5	0.5	0.4

[3] Abbreviations employed in this paper will be as follows: EDTA, ethylenediamine-tetraacetic acid; TRIS, tris-hydroxy-methyl-amino-methane; Succ, succinate; cyt c, cytochrome c; DDC, sodium diethyldithiocarbamate; Cyst, cysteine.

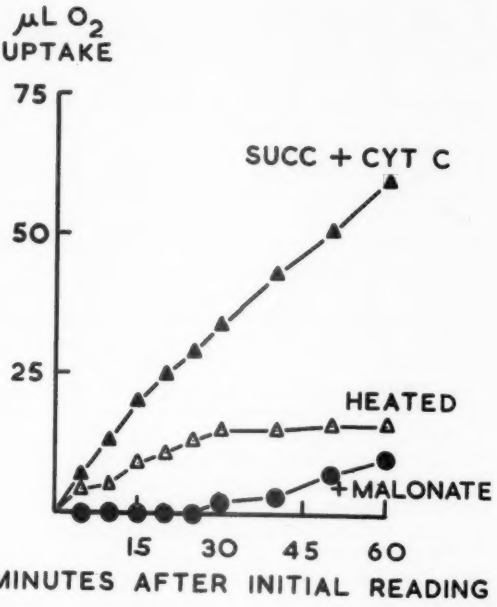

FIG. 1. Malonate inhibition of particle succinoxidase from roots of 2-day-old seedlings. Control heated in boiling water for 1 min. Conc of addenda in 2.00 ml and corresponding conc in the homogenizing medium in brackets: particles 22.3 mg/ml (44.6); K-succ 2.00×10^{-2} M (2.00×10^{-3}); cyt c 6.5×10^{-6} M (1.3×10^{-5}); pH 7.5 phosphate buffer 1.00×10^{-2} M (1.00×10^{-2}); cyst · HCl 5.00×10^{-3} M (1.00×10^{-3}); EDTA 5.00×10^{-4} M (1.00×10^{-3}); K-malonate 2.00×10^{-2} M; and sucrose to adjust medium to 0.5 osmolar (0.6).

Maintenance of the homogenate near pH 7 was essential for consistent demonstration of succinoxidase and cytochrome oxidase. A relatively high concentration of phosphate buffer (0.1 M) was used in the homogenization and assay media. The homogenates otherwise would have been acidic owing to the presence of EDTA, cysteine and vacuolar contents. Final pH values of the assay medium were about 7.

Results reported are not necessarily from experiments employing the formulations given in table I. Details are given as required.

ASSAY FOR ENZYMATIC ACTIVITY: Conventional Warburg manometric techniques were used to assay for enzymatic activity at 25° C.

In general, as the amount of enzyme preparation was limited, cytochrome oxidase assays were carried out in the same vessels and with the same preparations as used for the succinoxidase assay. One ml of enzyme extract was used in the initial 4.00 ml reaction mixture for the succinoxidase assay. After a reaction period, 0.5 ml of TRIS-neutralized ascorbic

acid solution was added from the side-arm for the cytochrome oxidase assay. The amount of ascorbic acid was sufficient to reduce 200 μl O_2.

The succinoxidase system is presumed to have been present when the oxygen uptake of reaction mixtures containing exogenous succinate was increased over controls without succinate (fig 2). Additional evidence that the system studied was succinoxidase came from a malonate inhibition of oxygen uptake (fig 1). The initial activity of the heated control in the experiment in figure 1 can be explained by insufficient heating because the particle suspension in a chilled tube was heated only for one minute in boiling water. In other experiments complete lack of succinoxidase activity was found for suspensions heated for longer periods.

Cytochrome oxidase is presumed to have been present when the oxygen uptake of reaction mixtures containing cytochrome c was greater than that of mixtures without cytochrome c (see fig 2 after ascorbate addition). Lack of cytochrome oxidase is not necessarily shown if cytochrome c did not stimulate activity. A more detailed discussion will follow.

Specific activities are reported on the basis of microliters O_2 uptake per hour per mg residue fresh weight, here designated Q_{O_2}. Various types of packing of spherical particles in the residue would allow 50 to 30 % of the packed volume to have been liquid. Consequently, the Q_{O_2} is an underestimate of activity per particle weight. Since the homogenizing medium contained nitrogenous compounds, specific activities based on nitrogen were approximate. When corrected for the nitrogen content of the medium activity per hour per mg particle nitrogen, ($Q_{O_2}(N)$) is about $100 \times Q_{O_2}$. All values reported are computed from total oxygen uptakes, i.e., uncorrected for autoxidation and endogenous activity.

Results

Succinoxidase: In early experiments succinoxidase was demonstrated only rarely in the particles isolated from barley root homogenates when the media used for extraction were composed of sucrose, sucrose plus phosphate, glucose plus phosphate, or variation of these with EDTA and protein. On the other hand, cytochrome oxidase was demonstrated. A report concerning an apparent functional succinic dehydrogenase in intact tissue but not in extracts (3) illustrates the difficulty in its demonstration in vitro in some plant extracts. The functioning in vivo of succinic dehydrogenase was shown in barley roots (16, 17).

Thus, the part of the succinoxidase system sensitive to damage was considered to be succinic dehydrogenase which is known to be an enzyme with essential sulfhydryl groups inactivated by RSSR compounds. Reactivation can be achieved with RSH compounds (9). Incorporation of the RSH compound cysteine into the homogenizing medium resulted in consistent demonstration of succinoxidase and cytochrome oxidase. Typical activities for succinoxidase were $Q_{O_2}(N) = 192$, and for cytochrome oxidase $Q_{O_2}(N) = 379$.

A marked oxidation of cysteine by the cytochrome system may have been indicated by the additional oxygen uptake of reaction mixtures with cytochrome c but without succinate. This was undesirable as cysteine oxidized by cytochrome c to cystine, or the free radical, inhibits succinic dehydrogenase (1). Incorporation of EDTA into the medium was accompanied by a lowering to the control level, of oxygen uptake of reaction mixtures with cytochrome c. The catalytic effect of cytochrome c in the cytochrome oxidase assay was unaffected (fig 2). From all experi-

Table II

Correlations between Initial Q_{O_2} and Particle Concentration in the Succinoxidase and Cytochrome Oxidase Assays

Assay	Measurements *	Treatment			
		Cyst	Cyst + cyt c	Cyst + succ	Cyst + succ + cyt c
Succin-oxidase	Coef of correlation	-0.04 ± 0.22 **	$+0.24 \pm 0.26$ **	$+0.05 \pm 0.20$ **	-0.02 ± 0.28 **
	Coef of regression, Q_{O_2}/mg × ml	−0.02	+0.04	+0.02	−0.01
	Extrapolated Q_{O_2}	1.12	0.60	1.33	2.67
	Mean Q_{O_2}	0.86 ††	1.10 ‡	1.60 ††	2.51 ‡
	Standard error of estimate, Q_{O_2}	0.65	1.01	0.86	1.00
	No. of cases	21	13	28	15
Cyt oxidase	Coef of correlation	-0.28 ± 0.20 **	-0.77 ± 0.10 †	-0.67 ± 0.12 †	-0.79 ± 0.07 †
	Coef of regression, Q_{O_2}/mg × ml	−0.03	−0.12	−0.05	−0.23
	Extrapolated Q_{O_2}	1.93 ‡‡	5.66 ‡‡	2.61 ª	6.59 ª
	Standard error of estimate, Q_{O_2}	0.60	0.80	0.42	1.12
	No. of cases	22	18	20	29

Assay mixtures contained EDTA. Treatments in the cytochrome oxidase assay all contained ascorbic acid.
* All values corrected for number of cases.
** Not significant at $P = 0.05$.
† Significant at $P < 0.01$.
††, ‡, ‡‡, ª Pairs of figures with the same symbol significantly different from each other at $P \leqslant 0.002$.

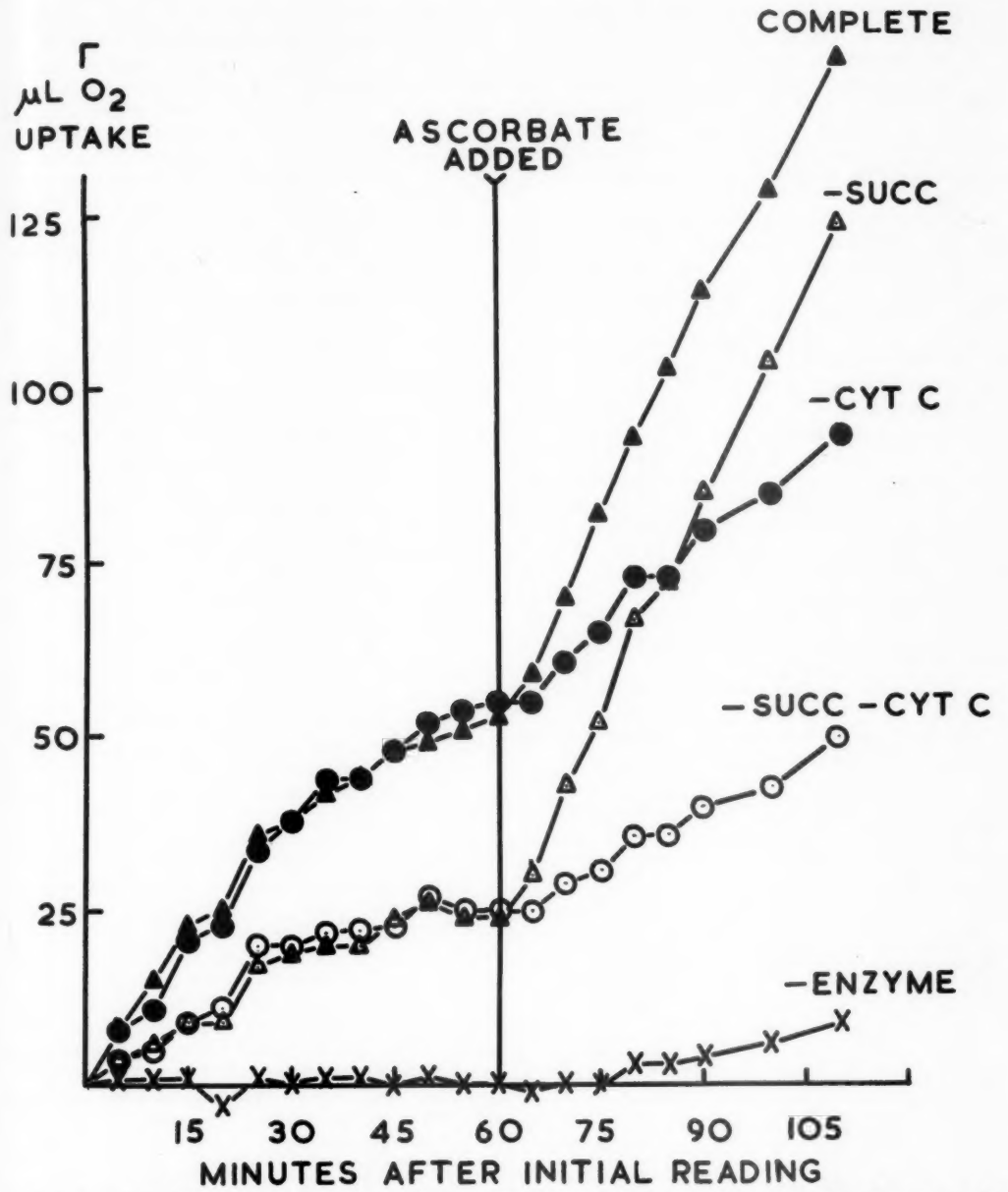

FIG. 2. Assay for succinoxidase and cytochrome oxidase with the same particle preparation from roots of 2-day-old seedlings. Conc of addenda in 2.00 ml for succinoxidase assay and corresponding conc in the homogenizing medium in brackets: particles 17.0 mg/ml (35.1); K-succ 2.00×10^{-3} M; cyt c 2.6×10^{-5} M; pH 7.5 phosphate buffer 6.00×10^{-2} M (1.00×10^{-1}); cyst·HCl 1.00×10^{-3} M (2.00×10^{-3}); EDTA 5.00×10^{-4} M (1.00×10^{-3}); and sucrose to adjust medium to 0.5 osmolar (0.6). Conc of K-ascorbate in 2.30 ml for cytochrome oxidase assay were 1.16×10^{-2} M.

ments with EDTA and without succinate, the mean Q_{O_2} with cytochrome c (table II, column 2) was not significantly different (P = 0.05) from treatments without cytochrome c (column 1) in the succinoxidase assay.

Succinoxidase activity decreased with time, especially in experiments employing a low concentration of particles per reaction volume (below 10 mg/ml). In experiments with dilute suspensions usually only the reaction mixtures containing succinate plus cyto-

chrome c showed initial activity. For all experiments with cysteine and succinate present (table II, columns 3 and 4), the effect of cytochrome in stimulating initial succinoxidase activity was significant ($P = 0.002$). Sustained succinoxidase activity was achieved by increasing the particle concentration. Although cytochrome c at the concentrations applied stimulated the activity of dilute suspensions, no sustained activity resulted.

Cytochrome Oxidase: Although cytochrome oxidase was consistently demonstrated, reproducible specific activities were difficult to obtain. A dilution test was desirable to determine whether the variations in activity were a result of dilution (loss of a factor) or of inhibitor action. Since a limited amount of particles could be prepared for any one experiment, usually no preparation was available for dilution tests after samples were allotted for the minimum number of controls. Consequently, the relation of specific activity to concentration of particles was determined from the correlations of results of several experiments. Data for correlations and regression lines are in table II and figure 3. Cytochrome oxidase activity (assayed with cytochrome c) was negatively correlated with particle concentration. Succinoxidase activity was not significantly correlated. For cytochrome oxidase a decrease of 0.12 to 0.23 Q_{O_2} per mg/ml increase in particle concentration was found (table II). It is inferred that some inactivating substance remained with the particle even after a wash treatment in the cold.

The results in table II also show that, in the cytochrome oxidase assay, the activity with cysteine and succinate (column 3) was significantly correlated, negatively, with particle concentration. However, the importance of the correlation is small since the coefficient of regression was low; i.e., the degree of inhibition by increased particle concentration, although significant, was small for the relatively low activity. Moreover, the Q_{O_2} value extrapolated to infinite dilution for the cysteine plus succinate treatment was not significantly different ($P = 0.05$) from the cysteine treatment. It may be concluded that the particle concentration was of importance only for the cytochrome oxidase stimulated by cytochrome c.

The addition of ascorbic acid to the particles resulted in real increases of oxygen uptake even in suspensions without cytochrome c in the cytochrome oxidase assay (figs 2 and 4). The extrapolated Q_{O_2} values in the cytochrome oxidase assay were significantly greater ($P \leqq 0.0001$.) than those in succinoxidase assay (table II, columns 1 and 3). Since the oxygen uptakes were greater than could be accounted for by autoxidation of ascorbic acid, the activity may have been due to ascorbic oxidase or cytochrome oxidase through endogenous cytochrome c. Thus, lack of cytochrome c stimulation is not necessarily evidence for lack of cytochrome oxidase in these assays. Since cytochrome c had significant stimulating effects (table II) the ambiguity was not of concern.

Succinoxidase and Cytochrome Oxidase from Older Roots: The methods used for 2-day-old seedlings were applied to roots of 10-day-old seedlings. Again both succinoxidase and cytochrome oxidase were found with particles isolated from the roots (fig 4). Rapid inactivation of the succinoxidase system occurred in this experiment, presumably owing to the low particle concentration used. This was also observed for preparations from 2-day-old seedlings and thus was not peculiar to the older tissue extracts. The small amount of succinoxidase activity was always observed when cytochrome and succinate were added to dilute particle suspensions. Moreover, the initial specific activity was substantially the same as that in experiments with higher particle concentrations which sustained the activity. Specific activity of cytochrome oxidase was as high as that of particles isolated from 2-day-old roots. In general, there was no detectable enzymatic difference between the particles from roots of 2-day-old seedlings and particles from roots of 10-day-old seedlings.

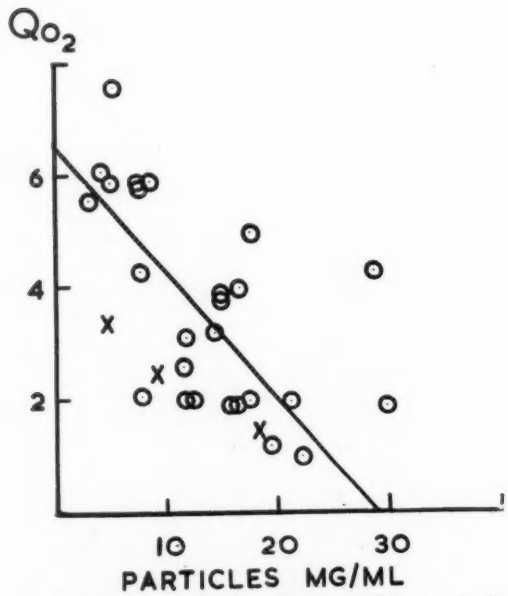

Fig. 3. Relation of initial Q_{O_2} to particle conc in the cyto oxidase assay. Assay medium contained cyst, succ, ascorbate, and cyt c. (See column 4 table II.)

Discussion

It was found that both succinoxidase and cytochrome oxidase are extractable from roots of barley seedlings as old as 10 days. Since these findings are contrary to those previously reported (10, 12), the method of demonstration will be discussed.

Succinoxidase: It was considered necessary to stabilize the labile succinic dehydrogenase of the succinoxidase system. Since cysteine treatment allowed activity to be demonstrated, it is inferred that RSH inactivators are released upon homogenization of the barley roots. Evidently only if appropriate steps to

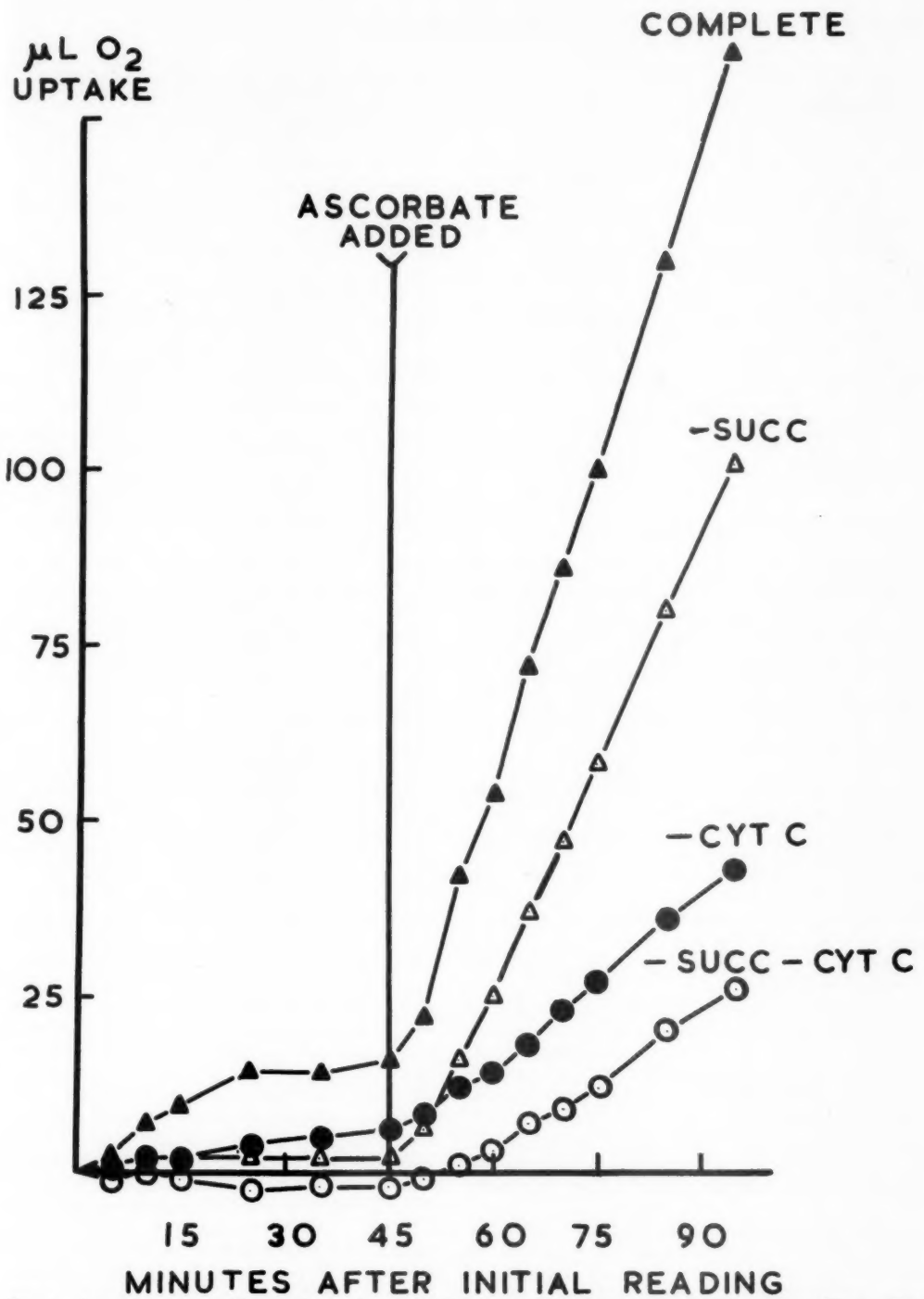

FIG. 4. Assay for succinoxidase and cytochrome oxidase of particles from roots of 10-day-old seedlings. Conc of addenda in 4.00 ml for succinoxidase assay and corresponding conc in the homogenizing medium in brackets: particles 6.0 mg/ml (24.1); K-succ 2.00×10^{-2} M; cyt c 6.4×10^{-5} M; pH 7.5 phosphate buffer 1.00×10^{-2} M (1.00×10^{-2}); cyst·HCl 2.50×10^{-4} M (1.00×10^{-3}); EDTA 2.50×10^{-3} M (1.00×10^{-2}); and sucrose to adjust medium to 0.5 osmolar (0.5). Conc of TRIS-ascorbate in 4.50 ml for cytochrome oxidase assay was TRIS 4.44×10^{-3} M and ascorbic acid 3.98×10^{-3} M.

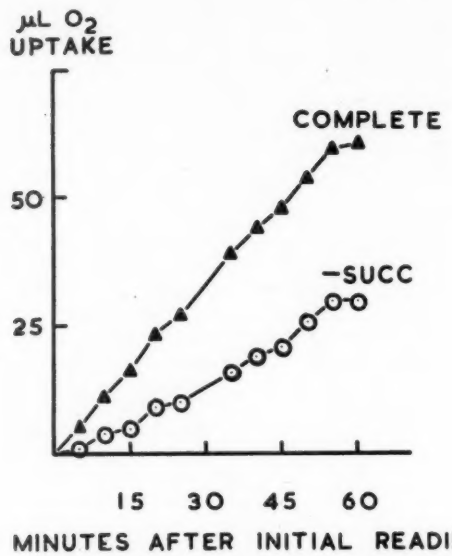

FIG. 5. Sustained succinoxidase activity of particles from roots of 10-day-old seedlings. Conc of addenda in 4.00 ml for succinoxidase assay and corresponding conc in the homogenizing medium in brackets: particles 9.4 mg/ml (18.8); K-succ 2.00×10^{-2} M; pH 7.5 phosphate buffer 1.00×10^{-2} M (1.00×10^{-2}); cyst·HCl 2.50×10^{-3} M (1.00×10^{-2}); EDTA 2.50×10^{-4} M (1.00×10^{-2}); and sucrose to adjust medium to 0.5 osmolar (0.5).

counteract the inactivation were taken, could succinoxidase be demonstrated consistently. Either the barley root succinic dehydrogenase may be more sensitive or there may be more RSH inactivator in barley roots than in potato tuber or cauliflower buds where the dehydrogenase is more easily demonstrated. For extracts from these latter two tissues appropriate steps to counteract apparent RSH inactivators increased oxidative activity but were not necessary for activity (18, 26).

Although a succinoxidase system was assumed to be evidence for the classical cytochrome oxidase (8) this is not necessarily so. Succinate oxidation mediated by a system presumably not involving cytochrome c oxidation has been observed (6). The system from barley roots when highly diluted showed activity, although short lived, only in the presence of cytochrome c. Therefore it appears that the succinoxidase system of barley roots includes cytochrome oxidase.

CYTOCHROME OXIDASE: Cytochrome oxidase itself was demonstrated using ascorbic acid as reducing agent. Since ascorbic oxidase is present in barley roots a small amount of contamination could account for ascorbate oxidation by particles from barley roots. However, the threefold stimulation of oxygen uptake by addition of cytochrome c showed that a large portion of the activity was mediated by cytochrome oxidase. In addition, the presence of cytochrome oxidase was confirmed with hydroquinone as reducing agent. The Q_{O_2} of particles assayed with the medium in table I (hydroquinone replacing ascorbate and without cysteine, insulin, and succinate) was 4.34 and 0.00 with and without cytochrome c, respectively.

Slater showed that non-enzymatic oxygen uptake was inhibited by cytochrome c when ascorbate was the reducing agent in a manometric assay for cytochrome oxidase. In addition, the presence of enzyme preparation lowered the "blank" correction (27). These data presumably indicate that non-enzymatic catalysis of ascorbic acid oxidation by metal ions is reduced by non-specific binding of the metal ions by protein. Oxygen uptake without barley root particles was zero with EDTA or, if not zero, was much lower than oxygen uptake by particles with no added cytochrome c and succinate. Particles, in contrast to Slater's results, therefore produced greater oxygen uptake than the non-enzymatic oxygen uptake. Further, both heating and malonate treatment lowered oxygen uptake of particles in the succinoxidase assay. Consequently, it is inferred that the oxygen uptake without exogenous succinate and cytochrome c was due to particle oxidation of endogenous substrate and cysteine. It is further inferred that this oxidation proceeded through a particle-bound cytochrome c system since the requirement for cysteine for endogenous activity paralleled its requirement for succinoxidase activity.

The inhibitory effect of high particle concentration was apparently specific to cytochrome oxidase and affected the succinoxidase system only slightly. The succinoxidase system may have been already rate limited, possibly by succinic dehydrogenase, as might be inferred from figure 2 (cf. Chance and Smith, 5). Consequently partial inhibition of excess cytochrome oxidase may not have markedly affected the succinoxidase system. Since succinoxidase was inactivated during the course of the reaction and cytochrome oxidase was not, even though its activity was assayed as much as one hour later, and since increased particle concentration sustained succinoxidase activity while it depressed cytochrome oxidase activity, it is obvious that a full explanation is complex. The relationship of high particle concentration to low specific activity may account partially for previous failures in isolating cytochrome oxidase from barley roots.

CYTOCHROME OXIDASE FROM OLDER ROOTS: It is known that barley roots exhibit a salt respiration (22). From the close correlation between cytochrome oxidase and salt accumulation and salt respiration it might be expected that cytochrome oxidase is active in barley roots. It was demonstrated here that succinoxidase and cytochrome oxidase are extractable from roots of both 2- and 10-day-old seedlings. The presence of cytochrome oxidase is consistent with but not sufficient to prove its activity in intact tissue.

James (12) suggested that cytochrome oxidase is replaced in older barley roots by ascorbic oxidase as the terminal oxidase. However, the evidence for James's conclusion was not unequivocal. The nega-

tive evidence of the lack of extractable cytochrome oxidase is now removed. The conclusion of James now depends largely upon the use of diethyldithiocarbamate (DDC) as a specific inhibitor of ascorbic oxidase in vivo. James and Garton (13) have shown the differential effect of DDC upon extracted ascorbic oxidase and extracted cytochrome oxidase. Nonetheless, the application of DDC to intact tissues cannot be considered to give conclusive results. It was confirmed that DDC markedly inactivates ascorbic oxidase in vivo.[4] However, the partial inhibition of respiration may not depend on the partial inactivation of ascorbic oxidase. Enough active ascorbic oxidase to accommodate the entire uninhibited respiration was extracted from 10-day-old barley root tips treated with DDC.[4] DDC can be oxidized to give a product which markedly inhibits succinic dehydrogenase (14). It is known that malonate, an inhibitor of succinic dehydrogenase, can reduce the oxygen uptake of barley root segments by 65 % (16), the same level of inhibition by DDC under similar conditions (13).

Summary

A method for the isolation and assay for particle-associated succinoxidase and cytochrome oxidase from barley roots is described. Some critical factors for the successful isolation of succinoxidase are the incorporation of cysteine into the homogenizing medium, maintenance of the pH near 7 during extraction and assay, and the avoidance of a dilute particle suspension in the assay. Although cytochrome oxidase is relatively easy to demonstrate, the final concentration of particles in the assay is important. An inhibitor of cytochrome oxidase which is retained even with the washed preparations was found to be associated with the particles.

In contrast to previous reports of unsuccessful attempts to demonstrate cytochrome oxidase in barley roots, cytochrome oxidase and also succinoxidase have been isolated from roots of both 2- and 10-day-old seedlings of Cape barley.

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[4] Honda, S. I. Unpublished experiments.

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EFFECTS OF ANTIMETABOLITES OF NUCLEIC ACID COMPONENTS ON THE GROWTH OF DIPLODIA NATALENSIS[1,2]

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Since the discovery that structural analogs of compounds of biological importance can interfere with enzyme systems and with growth, the use of antimetabolites for elucidating metabolic pathways has become widespread (16). In recent years antimetabolites have been used with considerable success in the study of the relation of nucleic acid synthesis to the metabolism and growth of bacteria and tissues of animals and higher plants. These methods, however, have not been employed extensively with fungi.

This paper will report the results of a preliminary survey of the effects of some analogs of purine and pyrimidine constituents of nucleic acids on the growth of *Diplodia natalensis* and attempts to reverse these inhibitions with the normal bases. Some attention was also paid to the interrelations of folic acid analogs and purine and pyrimidine bases to growth.

Materials and Methods

Diplodia natalensis Pole-Evans (U.S.D.A. strain 4725, the asexual stage of the ascomycete, *Physalospora rhodina*) was used as the test organism. It was grown on the following medium: glucose, 10 gm; asparagine-HCl, 2.0 gm; K_2HPO_4, 1.0 gm; $MgSO_4 \cdot 7H_2O$, 0.5 gm; Hoagland's micronutrient supplement, 1 ml; agar, 20 gm; water to 1 liter; pH 7.0. Inoculated Petri dishes were incubated for 3 days at $24° \pm 2°$ C at which time disks 5 mm in diameter were cut from the advancing edge of the mycelium with a sterile cork borer. In all experiments, a liquid medium was used having the same composition as above minus agar.

In these experiments, the tested purines and pyrimidines, folic acid, and reputed antimetabolites of these compounds were prepared as concentrated stock solutions and added to the liquid medium to give the reported final concentrations. The media were sterilized by autoclaving. Fifty ml of the liquid medium in a 250-ml Erlenmeyer flask was inoculated with a disk of mycelium and incubated for 7 days at 24° C. In every experiment 3 replicates of each variable were used. Mycelial mats were collected on filter paper circles in a Buchner funnel and were dried to constant weight at 80° C. The weight of the mycelium in unsupplemented control flasks was 192 mg ± 38 mg. Data are reported as percentages of the average weight of control cultures. Since the standard deviation of any reported value was ca 10 %, reported values differing from one another or from their appropriate controls by more than 25 % in any given experiment, may be considered significant.

Results and Discussion

Purine Analogs: The only noteworthy feature of the effects of adenine and guanine was the inhibition of growth caused by 0.01 M adenine (table I). Neither 2,6-diaminopurine nor benzimidazole, at the concentration tested, were highly inhibitory in contrast to the effect of 8-azaguanine. The fact that 2,6-diaminopurine relieved the inhibition caused by the superoptimal concentration of adenine suggests that it may do so by reducing the physiological level of adenine. The inhibitions of growth caused by the purine antimetabolites were, in all cases, almost completely reversed by high concentrations of either adenine or guanine. The reversals by the normal bases of the inhibitions caused by 8-azaguanine appear to be functions of the concentrations of the metabolites.

In two other ascomycetes previously examined, an

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TABLE I

EFFECT OF PURINES AND ANTIPURINES ON THE GROWTH * OF DIPLODIA

PURINES		ANTIPURINES (1×10^{-4} M)		
		2,6-DIAMINOPURINE	8-AZAGUANINE	BENZIMIDAZOLE
None	100	87	27	75
Adenine				
1×10^{-4} M	102	89	74	87
1×10^{-3} M	107	96	89	86
1×10^{-2} M	71	97	89	75
Guanine				
1×10^{-4} M	102	87	73	91
1×10^{-3} M	101	100	81	103
1×10^{-2} M	100	110	96	121

* Expressed as % of unsupplemented control (100).

adenineless mutant of Neurospora was inhibited by 2,6-diaminopurine and the inhibition reversed by adenine but not by guanine (4). In Ophiostoma, 2,6-diaminopurine could be utilized by a guanineless strain but not by an adenineless strain (6). In spite of the differences in the responses of these strains of Neurospora and Ophiostoma, both authors concluded that adenine and guanine were not interconvertible. For the alga, *Stichococcus subtilis* (1), 8-azaguanine inhibition was readily reversed by guanine. In *Tetrahymena gelii* (9) and *Lactobacillus casei* (2), 2,6-diaminopurine was able to satisfy completely the entire purine requirement. For *Saccharomyces cerevisiae, Endomyces vernalis* (17) and *Neurospora sitophila* (18), benzimidazole proved to be a powerful inhibitor of growth.

In yeast, this inhibition was relieved by both adenine and guanine although guanine was less effective than adenine. Hypoxanthine relieved the benzimidazole inhibition in Neurospora but adenine and guanine were not tested. For Diplodia, adenine and guanine may be substituted in vivo for one another since the inhibitions of the growth of Diplodia by two of the tested antipurines were reversed by either adenine or guanine.

PYRIMIDINE ANALOGS: Among the pyrimidine bases tested, orotic acid severely inhibited the growth of Diplodia (table II). Cytosine, thymine, and uracil appeared to be only slightly toxic in all tested concentrations. Among the three thiol-pyrimidines, only thiouracil induced severe inhibitions of growth; 5-aminouracil and 4-methylpyrimidine were only moderately inhibitory. Thiocytosine, itself not inhibitory, caused suppression of growth in the presence of 0.01 M cytosine, thymine, uracil and orotic acid. The suppression of growth caused by the lower concentrations of orotic acid was, however, relieved by thiocytosine. Thiothymine, on the other hand, reversed the slight inhibitions caused by all of the tested normal pyrimidine bases. The severe inhibition of growth caused by thiouracil was relieved by the normal pyrimidine bases in all concentrations tested. With the exception of orotic acid, the pyrimidine bases reversed the inhibitions caused by 5-aminouracil and 4-methylpyrimidine. The effects on growth caused by orotic acid were not modified by the presence of either of these antipyrimidines.

It is of interest to compare the interrelations of cytosine, thymine and uracil with their thiol-analogs. Although thiocytosine and thiothymine were ineffective in suppressing the growth of Diplodia, thiouracil was a potent inhibitor and uracil was the least inhibitory of the three bases. At the highest concentrations

TABLE II

EFFECTS OF PYRIMIDINES AND ANTIPYRIMIDINES ON THE GROWTH * OF DIPLODIA

PYRIMIDINES		ANTIPYRIMIDINES (1×10^{-4} M)				
		THIOCYTOSINE	THIOTHYMINE	THIOURACIL	5-AMINOURACIL	4-METHYLPYRIMIDINE
None	100	96	96	7	65	77
Cytosine						
1×10^{-4} M	86	96	92	88	79	72
1×10^{-3} M	80	89	113	71	99	88
1×10^{-2} M	77	66	117	71	93	108
Thymine						
1×10^{-4} M	90	80	91	78	100	102
1×10^{-3} M	86	86	101	70	107	100
1×10^{-2} M	80	69	114	89	84	78
Uracil						
1×10^{-4} M	90	73	98	87	86	66
1×10^{-3} M	89	78	107	83	98	74
1×10^{-2} M	93	88	101	107	88	100
Orotic acid						
1×10^{-4} M	64	91	97	88	79	78
1×10^{-3} M	73	98	102	89	96	77
1×10^{-2} M	32	17	61	28	43	31

* Expressed as % of unsupplemented control (100).

tested, uracil was the most effective in reversing the thiouracil inhibition, but at lower concentrations, cytosine and thymine were as effective as was uracil. For many microorganisms, the thiol-pyrimidines have been shown to be effective inhibitors of growth (3, 10, 19) and these inhibitions were reversible by the corresponding pyrimidine base. In some systems a particular thiol-pyrimidine was only antagonized by the corresponding metabolite (12) but in others (10) uracil reversed the inhibitions caused by all three thiol-derivatives. Thiouracil, the most active antipyrimidine for Diplodia was utilized, albeit poorly, as the sole source of nitrogen by a strain of *Neurospora sitophila* (18).

For certain bacteria (14) and fungi (11) orotic acid could satisfy the pyrimidine requirement in whole or in part. Diplodia, however, was inhibited by even relatively low concentrations of this compound. These inhibitions of growth were relieved by 5-aminouracil and the thiol-pyrimidines (table II), themselves growth inhibitors, as well as by uracil and thymine (table III). No explanation has been found to account for these results.

Folic Acid Analogs: Folic acid at 0.01 M had a slight inhibitory effect on the growth of Diplodia but 0.001 M produced no perceptible inhibition (table IV). Aminopterin and amethopterin were severely inhibitory and these inhibitions were relieved to a small extent by folic acid. A mixture of adenine, guanine, thymine, cytosine and uracil, each at 0.001 M, was considerably more effective in reversing these inhibitions. To investigate this phenomenon, each of these bases was tested separately in cultures containing 0.0001 M aminopterin. Adenine and guanine at 0.001 M each completely reversed the inhibitory effects of aminopterin. Uracil and thymine were also effective, uracil being more active than thymine. Cytosine, in contrast to the other pyrimidines, was completely ineffectual on the inhibited cultures. These findings have been confirmed in a separate experiment.

It has been demonstrated in animal tissues (7, 13) and in yeast (15) that aminopterin and amethopterin prevent the incorporation of formate or glycine into nucleic acid and coenzyme purines by interfering with formate incorporation into 4-amino, 5-imidazole carboxamide. Under these conditions, the carboxamide accumulated in the cells. In Ophiostoma, the carboxamide could be utilized by purine-requiring mutants (5). For Diplodia, adenine and guanine were, as might be expected, capable of reversing the aminopterin inhibition by by-passing the blocked step in purine synthesis. No adequate explanation has been found to account for the ability of uracil and thymine, but not cytosine, to reverse the inhibition of growth caused by aminopterin.

Table III

Inhibition of the Growth * of Diplodia by Orotic Acid and Its Reversal by Pyrimidines

Pyrimidines		Orotic acid (1×10^{-4} M)
None	100	51
Uracil		
1×10^{-6} M	82	88
1×10^{-5} M	96	72
Thymine		
1×10^{-6} M	69	82
1×10^{-5} M	95	91

* Expressed as % of unsupplemented control (100).

Table IV

Effects of Folic Antimetabolites on the Growth * of Diplodia and Their Reversals by Folic Acid and by Purine and Pyrimidine Bases

Metabolite		Aminopterin (1×10^{-4} M)	Amethopterin (2.5×10^{-6} M)
None	100	10	0
Folic acid			
1×10^{-5} M	97	51	12
1×10^{-3} M	74	4	24
Mixture of bases **	96	80	31
Adenine			
1×10^{-5} M	93	94	...
1×10^{-3} M	97	65	...
Guanine			
1×10^{-5} M	97	90	...
1×10^{-3} M	97	79	...
Cytosine			
1×10^{-5} M	87	8	...
1×10^{-3} M	87	11	...
Uracil			
1×10^{-5} M	94	102	...
1×10^{-3} M	89	88	...
Thymine			
1×10^{-5} M	92	9	...
1×10^{-3} M	86	74	...

* Expressed as % of unsupplemented control (100).
** Adenine, guanine, cytosine, uracil, thymine, each 1×10^{-5} M.

The results reported in this preliminary survey do not, of course, provide rigid proof that the systems involving purine and pyrimidine compounds are as indicated. Hitchings et al (8) have stated: "It does not follow that every substance which relieves an inhibition . . . can be assumed to be a product or substrate of normal metabolism." Our data do, however, suggest several fruitful leads as to the direction which detailed work may take in order to elucidate the questions raised.

Summary

Purines, pyrimidines, folic acid and antimetabolites of these compounds were tested for their effects on the growth of a prototrophic strain of the fungus, *Diplodia natalensis*. Growth was severely inhibited by 8-azaguanine and the inhibition relieved by adenine and guanine. Thiouracil was the most potent antipyrimidine tested, and its effects could be overcome by cytosine, thymine, uracil, or orotic acid. Aminopterin and amethopterin were both highly in-

hibitory antimetabolites of folic acid. Folic acid, adenine, guanine, thymine, and uracil reversed these inhibitions. The implications of some of these findings are discussed.

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CHLOROPHYLL AND CAROTENOID DESTRUCTION IN THE ABSENCE OF LIGHT IN SEEDLINGS OF ZEA MAYS L.[1,2]

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Seedlings require light not only for the initial formation of chlorophyll, but apparently also for its continued maintenance, for a seedling containing chlorophyll will, when placed in the dark, lose its green pigmentation (1). This suggests that, to maintain a high chlorophyll level in the living plant, light-dependent synthetic reactions must keep pace with, or exceed the light-independent destructive processes that occur concomitantly and continuously. These "dark" destructive processes must occur in addition to the photochemical oxidations of the chlorophyll molecule extensively described in the literature (2, 3).

Carotenoid pigments can be synthesized by dark-grown seedlings, but under certain conditions light will accelerate the synthesis of these yellow plastid pigments (4, 5, 6, 7, 8). The destructive reactions of the carotenoids have also been studied in detail, and are considered to be both enzymatic and photochemical in nature (9).

Thus, it is evident that the amount of chlorophyll and carotenoids present in a plant grown in the light is the resultant of at least the following reactions:

1) "light" formation of chlorophyll,
2) "light" destruction of chlorophyll,
3) "dark" destruction of chlorophyll;

and for carotenoids,

1) "dark" formation,
2) "light" formation,
3) "dark" destruction,
4) "light" destruction.

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The present investigation was undertaken to elucidate those destructive reactions of chlorophyll and carotenoids that occur exclusively in the absence of light. In this way, it is hoped that the complex network of reactions involving these pigments will begin to be unraveled.

Materials and Methods

Materials: Seedlings of *Zea mays* L. of a hybrid stock named Cornell M-4 were used in this investigation.[4] Seeds were soaked for 24 hours in distilled water, and after soaking, about 25 seeds placed around a rim of a 600-ml beaker, between the glass surface and a layer of moistened filter paper. Distilled water (350 ml) was placed in each beaker to insure a high relative humidity. The beakers were placed in a constant temperature chamber at 27° C in the complete absence of light, and the seedlings allowed to develop until they were one week old. At the end of this time, a sample of plants was extracted to determine the concentration of carotenoid pigments that had developed during this period, some seedlings were left in the dark chamber to serve as controls, and the remaining seedlings were exposed to various conditions of light. After the exposure period, samples of the light-exposed seedlings were killed, extracted and analyzed for pigment content (chlorophyll and carotenoid), and the remaining light-treated plants in this series of exposure times were then returned to the dark 27° C temperature chamber. Seedlings were periodically sampled, after various times in the dark, and extracts made to follow the changes in chlorophyll and carotenoid content occurring during the dark period. At the same time, control plants were analyzed, that is, plants which were comparable in age, but which had received no light treatment.

Extraction Procedure: The seedlings were killed by placing them in boiling water for one minute, this treatment also serving to minimize the oxidative destruction of carotenoids (10). The sheath was dissected away, and the leaf tissue weighed on a torsion balance and immediately extracted. Four seedlings were used for each extract. The wet weights of the leaf tissue averaged from 100 to 800 mgs. The tissue was ground in a mortar containing Berkshire sand and 20 ml C.P. methyl alcohol. The grinding of the plant tissues was carefully timed and kept standardized. At the end of the grinding period, the extract was filtered with suction and the filtered tissue examined to see that it was colorless. The filtrate was then centrifuged for ½ hour, and the clear greenish-yellow methyl alcohol solution brought up to constant volume (25 ml) before measuring the pigment content spectrophotometrically.

Estimation of Pigment Content: The solutions were analyzed in a Beckman spectrophotometer Model DU. The photometric density at 660 mμ was taken as a measure of chlorophyll concentration, and the density at 440 mμ was used to estimate the total carotenoid concentration. In these seedlings, the chlorophyll concentration was always low relative to the carotenoid concentration, so the following method of estimating carotenoids was adopted: chlorophyll a has two peaks of absorption of almost equal height, one at 440 mμ, one at 660 mμ; the optical density of the solution at 660 mμ was subtracted from its optical density at 440 mμ, the remainder being considered a measure of the carotenoid concentration. The presence of chlorophyll b would introduce an error in this method of estimating carotenoid content, since the relative height of its absorption peak at 440 mμ compared with 660 mμ is 2.87. No evaluation of chlorophyll b concentration was made, but it can be estimated that its presence might cause at most a 10 % error in the carotenoid determination. (In the experiments reported here, the optical density of the extracts at 660 mμ was never more than $\frac{1}{3}$ the optical density at 440 mμ, and at most only $\frac{1}{3}$ of the density value at 660 mμ would be due to the presence of chlorophyll b.)

In all tables and figures, pigment concentration is expressed as density per mg wet weight of leaf tissue. However, the raw data were obtained by analyzing the pigment content in the leaves of 4 seedlings, whose fresh weights were known. Thus, the change in total pigment content in each seedling with time in the dark could also be followed. Light-treated plants invariably had higher fresh weights than dark-grown plants of comparable age, and it was felt that this increased growth might also involve increased pigment synthesis. In an effort to balance out this effect, the fresh weight basis for expressing pigment concentration was decided upon, rather than a per plant basis. However, pigment changes expressed on a per plant basis were essentially of the same order as those expressed on a per mg fresh weight basis. That is, the percentage of pigment loss with time in the dark was parallel in both cases. This is true in spite of the fact that plants returned to the dark showed increases in wet weight. A dilution of pigment concentration would be expected as a result. Evidently, this dilution effect was constant over the range studied here, since in spite of its presence the time course of pigment destruction was not different from that of pigment loss within a single seedling.

Light Exposure: The seedlings were exposed to white light for varying durations (from 4 to 12 hours) at a constant light intensity of 90 fc. The light source was a 300-watt projection bulb operating through a voltage regulator on 115 volts AC; the intensity of the light was measured using a Macbeth illuminometer. When the seedlings were about to be exposed to light, they were taken from the beakers and transferred between two glass surfaces to orient them uniformly to the light source. The exposure took place in a specially constructed light-tight box, the only light falling on the plants coming from the projection

[4] These seeds were made available for experimentation by the generosity of the Department of Plant Breeding, Cornell University Agricultural Experiment Station, Ithaca, N. Y. and are gratefully acknowledged.

bulb. At all other times, the plants were handled in the dark, with the aid of a dim green Christmas tree bulb as safe light.

Results and Discussion

Table I shows the effect of 4 different light exposures on the amount of chlorophyll and carotenoid pigment in the leaf tissue of one-week-old corn seedlings. The figures give an idea of the type of variability encountered.

In tables II and III and figure 1, the time course of the light-independent destruction of the two pigments is shown. Each figure in the table and point on the curve is an average of at least 5 determinations. Regardless of the initial pigment concentration in the seedling at the time of return to the dark, the rate of the pigment destruction is the same. After 120 hours in the dark at 27° C, virtually all the chlorophyll initially present is destroyed (upper curve, fig 1). The time course of chlorophyll destruction was followed in a few cases, not shown in table II

Table I

Effect of Different Durations of Exposure to White Light on the Chlorophyll and Carotenoid Content of 1-Week-Old Etiolated Corn Seedlings

Time of exposure in hrs	Pigment conc *	
	Chlorophyll ($\times 10^{-5}$)	Carotenoids ($\times 10^{-4}$)
4	19.0	5.2
	22.7	6.7
	14.7	4.2
	8.7	2.3
	averages	
	15.4	4.4
6	29.0	3.5
	27.1	5.3
	22.3	3.8
	32.5	6.2
	13.8	4.3
	17.9	4.7
	21.1	5.3
	averages	
	23.4	4.7
8	33.8	6.1
	28.0	5.6
	30.4	4.9
	30.9	4.8
	averages	
	30.8	5.4
12	43.4	5.2
	27.9	4.4
	31.6	4.3
	43.3	4.9
	29.6	4.5
	averages	
	35.2	4.5

* Pigment concentration is expressed as the optical density of the extracts/mg wet wt of leaf tissue, the density at 660 mμ measuring the chlorophyll conc, and the density at 440 mμ, the carotenoid conc.

Table II

Effect on the Chlorophyll Content of 1-Week-Old Corn Seedlings When Placed in the Dark at 27° C after Previous Light Exposure of Different Durations

Light exposure in hrs	Chlorophyll conc * ($\times 10^{-5}$)	Hrs in dark after light treatment	Chlorophyll conc * ($\times 10^{-5}$)	% Loss of pigment
4	15.4	24	8.3	47
		48	4.7	69
		72	4.2	72
		96	3.6	76
6	23.4	24	16.3	31
		48	10.2	56
		72	3.8	83
		96	5.2	77
		120	4.0	83
		144	2.2	90
8	30.8	24	20.7	33
		48	11.2	64
		72	6.7	78
		96	6.5	79
		120	5.0	84
		144	2.5	92
12	35.2	24	28.5	19
		48	13.4	61
		72	7.4	78
		96	4.7	86
		120	5.6	84
		144	3.9	88

* Chlorophyll conc is expressed as the optical density of the extracts at 660 mμ/mg wet wt of leaf tisue.

or figure 1, where the initial concentration of chlorophyll present in light-treated seedlings prior to return to the dark was 5 to 150 times greater than the lowest value in table II. Here too, the destruction of pigment proceeded in these seedlings at the same rate as in the other plants. Thus, the time required for the destruction of a given percentage of pigment is independent of the initial concentration, even though the initial concentration may vary 150-fold. Such a kinetic picture suggests that the destruction of chlorophyll proceeds as a first-order reaction where the rate at which the molecular species disappears is proportional at each instant to the amount of that species.

Unlike the chlorophyll destruction curve, the carotenoid destruction curve (lower curve, fig 1) does not approach 100 % loss of pigment but seems to level off at 40 % of the initial pigment concentration. A possible interpretation of this curve is suggested by an examination of table IV.

Table IV shows the light-independent destruction of carotenoids in seedlings previously light-treated compared with control seedlings of the same age, grown throughout their history in the dark. It is clear that carotenoids are destroyed with time in the dark in both the light-treated seedlings and in the dark-grown controls. From an examination of the rate of carotenoid destruction in the dark-grown control plants, it can be seen that this rate is slower than the dark destruction of chlorophyll. After 120 hours, about 50 % of the carotenoids have disappeared, while

TABLE III

EFFECT ON THE CAROTENOID CONTENT OF 1-WEEK-OLD CORN SEEDLINGS WHEN PLACED IN THE DARK AT 27° C AFTER PREVIOUS LIGHT EXPOSURE OF DIFFERENT DURATIONS

LIGHT EXPOSURE IN HRS	CAROTENOID CONC * ($\times 10^{-4}$)	HRS IN DARK AFTER LIGHT TREATMENT	CAROTENOID CONC * ($\times 10^{-4}$)	% LOSS OF PIGMENT
4	4.4	24	4.5	0
		48	3.6	18
		72	2.8	36
		96	2.4	41
6	4.7	24	4.5	4
		48	3.8	19
		72	2.3	51
		96	2.8	41
		120	2.7	43
		144	2.0	57
8	5.4	24	4.9	9
		48	3.4	37
		72	2.8	48
		96	2.5	53
		120	3.1	45
		144	2.1	61
12	4.7	24	4.8	0
		48	3.5	25
		72	2.8	40
		96	2.7	40
		120	2.5	46

* Carotenoid conc is expressed as the optical density of the extracts at 440 mμ/mg wet wt of leaf tissue.

table II indicates that for this time interval, well over 80 % of the chlorophyll has disappeared.

Column 3 of table IV shows the difference in amount of pigment in the "light" and "dark" plants at different times in the dark. This increment decreases very rapidly with time, and the difference all but disappears after 96 hours in the dark. Thus, actually, the "light-formed" carotenoids disappear completely after 96 hours, and the lower curve in figure 1 is deceptive. While it is true that the total carotenoid concentration is never completely depleted in the dark, as shown by this curve, an examination of column 3 of table IV shows that in reality the carotenoids formed by light are completely destroyed during the time interval of these experiments.

An alternative interpretation to these data, however, is that we are dealing with chlorophyll b disappearance rather than the destruction of light-synthesized carotenoids. The difference between the carotenoid destruction in the dark-grown and light-treated plants is of sufficiently small magnitude to make this alternative plausible.

EFFECT OF SUCROSE ON PIGMENT DESTRUCTION: A seedling growing in the light enjoys the benefits of continuous sugar production, whereas the seedlings in these experiments were returned to the dark with little food reserves, their source of energy being the small amount of sugar produced during the short light exposures and whatever may remain of food in the endosperm. The following question thus arises. Can

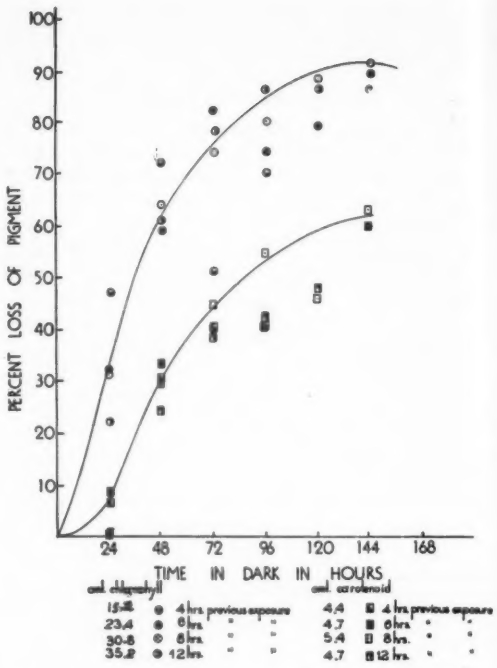

FIG. 1. The time course of the light-independent destruction of chlorophyll (*upper curve*) and carotenoids (*lower curve*). Numbers at the bottom show the concentration of chlorophyll and carotenoid initially present in seedlings at the time of return to the dark.

exogenous sucrose protect the "light-independent" destruction?

Tables V and VI and figure 2 show the effect of adding sucrose to the seedlings during their "dark" period following light exposure. In table V and figure 2, the effect of various concentrations of sucrose was determined for seedlings previously exposed to 12 hours of light and returned to the dark for 48 hours.

TABLE IV

LIGHT-INDEPENDENT DESTRUCTION OF CAROTENOIDS IN PREVIOUSLY LIGHT-TREATED CORN SEEDLINGS (12 HRS LIGHT EXPOSURE) AS COMPARED WITH DARK-GROWN CONTROLS OF THE SAME AGE

HRS IN DARK	CAROTENOID CONC * ($\times 10^{-4}$)		DIFFERENCE BETWEEN LIGHT-TREATED AND DARK CONTROLS
	PREVIOUSLY LIGHT-TREATED	DARK CONTROLS	
0	5.7	4.6	1.1
28	4.6	4.2	0.4
48	4.4	4.1	0.3
72	2.9	2.7	0.2
96	2.4	2.5	−0.1
120	2.3	2.2	0.1

* Carotenoid conc is expressed as the optical density of the extracts at 440 mμ/mg wet wt of leaf tissue.

TABLE V

EFFECT OF VARIOUS SUCROSE SOLUTIONS ON THE LIGHT-INDEPENDENT DESTRUCTION OF CHLOROPHYLL AND CAROTENOIDS

TREATMENT *	SUCROSE CONC %	CAROTENOID CONC ** ($\times 10^{-4}$)	% LOSS OF CAROTENOIDS	CHLOROPHYLL CONC ** ($\times 10^{-3}$)	% LOSS OF CHLOROPHYLL
12 Hrs light	0	4.5	..	29.6	..
12 Hrs light	0	2.8	37	8.2	72
+48 hrs	1	3.3	27	13.2	55
dark	2	3.95	11	16.7	43
	3	4.5	0	18.3	38
	4	4.7	0	19.5	34
	5	4.2	0	21.0	29

* One-week-old corn seedlings were previously exposed to 12 hrs white light and returned to the dark for 48 hrs.

** Pigment conc is expressed as the optical density of the extracts/mg wet wt of leaf tissue, the density at 660 mμ measuring chlorophyll conc, and the density at 440 mμ, the carotenoid conc.

The destruction of both chlorophyll and carotenoids was affected by the added sucrose. The carotenoids were completely protected by 3, 4, and 5 % sucrose solution (lower curve, fig 2), while the chlorophyll values showed approximately a 30 % drop for these sugar concentrations, as compared with 72 % loss of pigment when no sugar was added.

In table VI chlorophyll destruction in seedlings was followed for different times in the dark in the presence and absence of 2 % sucrose. The seedlings had been previously exposed to 12-hours light before their return to the dark. The added sucrose affected the destruction of chlorophyll, but the effect seems to be to delay the onset of the destruction rather than to prevent it. Chlorophyll is destroyed with time in the dark in both the sucrose and non-sucrose treated seedlings. However, the last column in the table

TABLE VI

EFFECT OF ADDING 2 % SUCROSE SOLUTIONS ON THE COURSE OF THE LIGHT-INDEPENDENT DESTRUCTION OF CHLOROPHYLL

HRS IN DARK AFTER PREVIOUS LIGHT TREATMENT	CHLOROPHYLL CONC *		% PIGMENT DESTRUCTION		DIFF IN DESTRUCTIVE RATES
	No SUCROSE	2 % SUCROSE	No SUCROSE	2 % SUCROSE	
0	36.5	...	..	..	..
48	21.9	25.2	40	31	9
72	7.5	11.9	79	67	12
96	4.7	8.9	87	75	12
120	5.6	10.0	84	72	12
144	3.9	3.1	89	91	−2

One-week-old corn seedlings, previously exposed to 12 hrs white light were returned to the dark at 27° C for various times.

* Chlorophyll conc is expressed as the optical density of the extracts at 660 mμ/mg wet wt of leaf tissue.

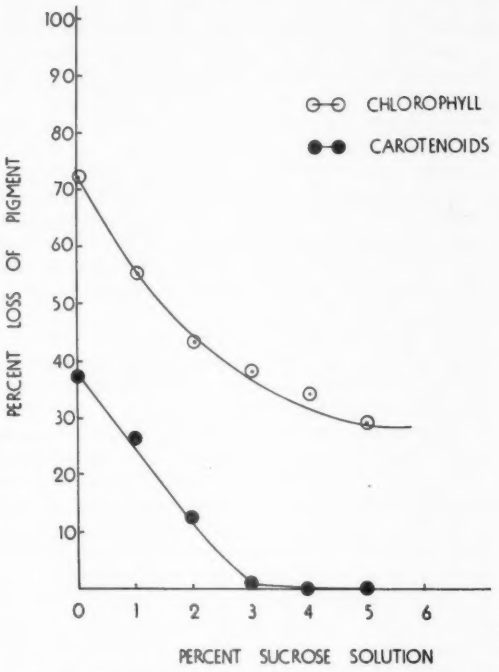

FIG. 2. The effect of adding sucrose solutions to seedlings previously light-treated for 12 hrs and returned to the dark for 48 hrs. *Upper curve.* The effect of sucrose solutions on the light-independent destruction of chlorophyll. *Lower curve.* The same for carotenoids.

shows the difference in destruction rates of the two groups. This difference is relatively constant for 120 hours in the dark, suggesting that the added sucrose has merely shifted in time by a constant amount the inevitable destruction of chlorophyll. Beyond the 120-hour period, the sucrose failed to affect the pigment destruction.

A similar type of study was made to test the effect of 2 % sucrose on carotenoid destruction. The results showed considerable variation, so they are not published here. In general, however, the sucrose effect seemed to be similar; that is, the carotenoids are destroyed in the presence and absence of sucrose, and there has been a shift in time of onset of this destruction in the sucrose-treated seedlings.

Just how the sucrose accomplishes this delay in the onset of pigment destruction can only be a matter of speculation. Perhaps sugar helps to maintain some plastid structure that temporarily binds the pigments in a stable structure.

It is clear, however, that depriving a plant of light and thus adequate sugar supply affects pigment maintenance in a way that is separate and distinct from the role of light and sugar in pigment synthesis. A young etiolated seedling, when exposed to light, forms chlorophyll from protochlorophyll. Protochlorophyll can be synthesized by the plant in the absence of

light, the light acting as the agent only in the ultimate step in chlorophyll formation. Sugar and other organic and inorganic compounds are presumably needed for the initial and continuous formation of protochlorophyll. A short time after chlorophyll has been formed, the plant becomes capable of photosynthesis and new sugars are thus supplied for further pigment synthesis. When such a seedling is deprived of light, chlorophyll formation is halted by the direct removal of the agent in the protochlorophyll-chlorophyll transformation; secondarily, sugar production ceases, and this affects the plant's ability to synthesize protochlorophyll, and other simpler precursors of the chlorophyll molecule.

We have seen here, however, that removing the plant from the light not only halts further pigment synthesis but affects the plant's ability to maintain the pigments it already has.

The seedling returned to the dark fails to maintain both chlorophyll and carotenoids. In earlier work with oat seedlings (6) we had shown that when chlorophyll was formed, the carotenoids fall in concentration, suggesting that the carotenoid molecule might be involved in the synthesis of the chlorophyll molecule, perhaps by supplying the phytol group. In the light-independent destruction of the chlorophyll molecule, if the backward reaction followed the same course as the forward reaction in reverse, one might expect to get an accumulation of carotenoids as the chlorophyll content decreased. However, the study reported here shows clearly that when chlorophyll is destroyed, carotenoids are also destroyed. So no reciprocal relationship exists between the two pigments with respect to their light-independent destruction.

Summary

One-week-old corn seedlings containing known amounts of chlorophyll and carotenoids were placed in the dark, and the time course of the destruction of the pigments followed. Both chlorophyll and carotenoids were destroyed rather rapidly at 27° C in the dark. All the chlorophyll disappeared completely after 120 hours in the dark, while the carotenoids fell to 40 % of their original value.

It was found that, even though the initial pigment concentration varied as much as 150-fold, the time required for the destruction of a given percentage of pigment was independent of the initial concentration. This suggests that the light-independent pigment destruction follows the kinetics of a first-order reaction.

Adding sucrose to the environment of the seedlings when placed in the dark effectively protected for a time against depletion of both chlorophyll and carotenoids. Concentrations of 3 % sucrose protected the carotenoids completely from destruction in the dark, whereas the chlorophyll concentration decreased 30 % in 3 % sucrose as compared with 72 % destruction in the absence of sucrose.

The protective effect of exogenous sucrose on pigment destruction seemed to be to delay the onset of destruction rather than to prevent it from occurring.

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RESPIRATION AND SALT ABSORPTION BY EXCISED BARLEY ROOTS[1,2]

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The fact that absorption of salt by plant tissue is usually accompanied by stimulation of respiration has been recognized for some twenty years.

Salt effects upon respiration have been studied in various types of plant tissue: wheat roots (8 to 10), carrot slices (15 to 20), beet slices (18), potato discs (23 to 25) and barley roots (11) among others. The phenomenon may then reasonably be believed to be of general occurrence.

In the early thirties Lundegårdh and Burström began a series of researches which culminated in an ingenious theory to account for the simultaneous absorption of salt and increase in the respiratory rate. The point of view of Lundegårdh and Burström has

[1] Received March 15, 1955.

[2] This paper is based on work performed under contract No. AT-(11-1)-34, project 5, with the Atomic Energy Commission.

been espoused with modification by Robertson and his co-workers.

The bases of this theory are well known and need not be presented here. In brief, it is proposed that oxidized cytochrome is capable of attracting anions from the external medium and that accumulation occurs either as a result of the inward movement of anions along a chain of stationary cytochrome enzymes or under Robertson's modification, of the inward diffusion of particles (mitochondria?) bearing the oxidized cytochrome-anion complex through a region in the tissue which is impermeable to free anions. At the inner boundary of this barrier, anions are released as reduction of the cytochrome occurs and outward diffusion of the particles begins. This is depicted schematically in figure 1.

The reaction occurring at the inside boundary (i.e., presumably at the tonoplast, if the model is assumed to represent a single cells) is as follows:

$$FH_2 + 2\,\text{Cyt Fe}^{+++} \longrightarrow 2\,\text{Cyt Fe}^{++} + 2\,H^+ + F$$

where F and FH_2 represent oxidized and reduced forms respectively of the flavoprotein enzyme. At the outer boundary the reoxidation of cytochrome occurs:

$$2\,H^+ + \tfrac{1}{2}\,O_2 + 2\,\text{Cyt Fe}^{++} \longrightarrow 2\,\text{Cyt Fe}^{+++} + H_2O$$

It should be noted that this scheme contains an assumption not readily susceptible of proof, namely that the loss of an electron consequent upon the oxidation of the iron of reduced cytochrome must be balanced by the acquisition of an anion. Of the components of the cytochrome system only cytochrome c has been isolated and characterized. Calvin and Martell (3) state that ferrocytochrome c is diamagnetic while the ferric form is paramagnetic, having one unpaired electron. The bonds of both forms are covalent and the only change taking place in the course of its biological action is the gain or loss of an electron. If, as the theory assumes oxidation of ferrocytochrome results in an unbalanced positive charge, the charge so produced may be balanced either by the absorption of an anion as shown in figure 1, or by the disappearance of H^+ produced in the substrate oxidation. In the former case, the H^+ utilized in the reoxidation of reduced cytochrome must come from another source, possibly the external medium as shown.

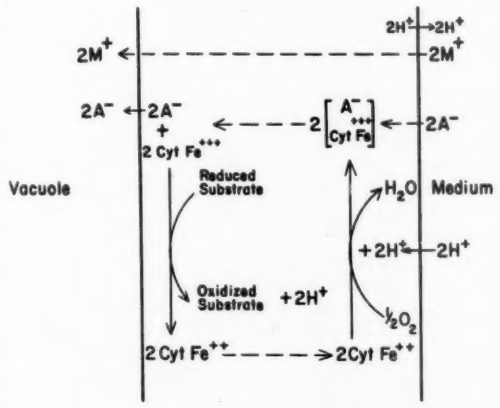


FIG. 1. Schematic representation of the Lundegårdh-Robertson hypothesis. M^+ = inorganic cation, A^- = inorganic anion.

According to the Lundegårdh view, increased respiration results from the stimulating effect of anions in general upon the activity of the cytochrome system. When anions are not provided in the external medium as in distilled water, the terminal oxidase becomes dependent upon endogenous anions presumed to be largely malate and is only very sluggishly operative.

Cations are viewed as being inert in this regard. They are presumed to pass readily through the salt barrier by a process of repeated exchange for H^+ derived from organic constituents of acid dissociation. Measurements of root surface potentials (9) indicate that the protoplasm is predominantly electronegative. The driving force for cation accumulation (fig 1) lies evidently in the production and utilization of H^+ at the inner and outer boundaries respectively.

Steward and his collaborators working in the thirties and early forties opposed the Lundegårdh hypothesis and presented evidence that, in potato slices at least, the cation plays a dominant role in the production of salt respiration as well as in other salt effects upon metabolism. In their work potassium ion was found to stimulate a number of metabolic processes: protein formation, water absorption, phenolase activity and hydrolysis of starch as well as respiration. Calcium ion was found to exert a depressing effect upon these same activities. The extent of these phenomena are clearly seen in the data of Steward et al (24).

Although postulating that the function of aerobic respiration in the salt absorption process lies in the provision of energy necessary for accumulation of ions against their diffusion gradients rather than in mere production of HCO_3^- and H^+ for exchange with other ions in the external medium, Steward and his co-workers did not speculate upon actual absorption mechanisms.

The present work was designed to investigate the phenomenon of salt respiration as it occurs in the material currently being used at Berkeley in ion absorption studies. In particular it was desired to learn whether or not the behavior of this material would support the postulation of a direct and causative link between the stimulation of respiration and the absorption process per se.

MATERIALS AND METHODS

All experiments in this study were carried out with the excised roots of Atlas barley grown from seed of the 1946 crop by the method of Jacobson and Overstreet (6). This method was modified slightly in that the preliminary treatment of the seed with H_2O_2 was eliminated. Respiration was determined as

O_2 absorbed. During the experimental period the excised roots were confined in glass stoppered, bubble-free reagent bottles completely filled with the appropriate solutions. The solutions to be used for any experiment were aerated for a period of 48 hours at 26° C prior to the start of the experiment.

Except where otherwise noted, a root to solution ratio of 3 gm/l was used, the weight of roots being calculated for each bottle according to its total capacity.

The bubble-free stoppered bottles containing roots were kept at 26° C ± 0.1° C during the experiment by means of a water bath. During this period the solutions were "stirred" by inverting the bottles several times every 5 minutes.

As the experimental period for each sample ended, the bottle was removed from the bath and duplicate samples were taken for analysis. These were collected in 250-ml reagent bottles, glass stoppered and calibrated for total volume. Collection was made using a siphon, the delivery end of which was kept below the level of liquid in the reagent bottle to avoid introduction of oxygen by splashing. The collection bottles were completely filled and stoppered immediately.

The difference in concentration of oxygen between the initial sample and that taken at the end of the experimental period was used to determine the O_2 absorbed; this was expressed as millimoles/kg fresh weight. Oxygen was determined by Winkler's iodometric method (22).

In this way very reliable results were obtained, the difference between duplicate samples being usually less than 0.2 %.

This procedure for measurement of respiration was adopted instead of the conventional Warburg method because it permits use of root solution ratios comparable to those heretofore used at Berkeley in ion absorption studies. Also it permits measurement of both absorption and respiration by the same root sample thus eliminating one source of variability.

In each case, where the effects of salts upon the respiration rate were being determined, a control determination of respiration in distilled water was made simultaneously in order to eliminate errors arising from differences between batches of roots. The average deviation from the mean respiration rate during a 13-week period amounted to 3.0 %.

After samples of solution had been withdrawn for measurement of O_2, the roots were immediately transferred to a nylon screen, thoroughly washed with distilled water and then transferred again to containers for drying preparatory to analysis for absorbed ions. In every case an initial sample of roots was also analyzed, the amount of a given ion absorbed being determined as the difference between the contents of initial and treated samples. Results of the analyses were expressed as meq absorbed/kg fresh weight.

Experimental Results

Preliminary Experiments: The method of measuring respiration and absorption in closed bottles outlined in the preceding section has not heretofore been widely employed. While it has the advantages previously mentioned, this procedure is obviously suitable only for measurements over a curtailed experimental period, since the tissue respires and absorbs salt in a medium of waning O_2 and waxing CO_2 concentration. It must therefore be expected that at some period after introduction of the roots into the bottle, the influence of one or both of these factors will cause a significant lowering of the absorption and respiration rates, which in an open system might remain constant for a much longer period.

In preliminary experiments using distilled water and solutions giving rise to a pronounced stimulation of respiration it was found that the respiratory rate became constant within 10 to 15 minutes after the root samples had been introduced into the bottles.

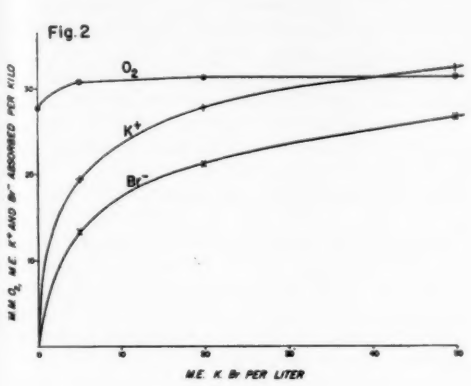
These experiments revealed also a significant slackening of the previously stable respiration rate after 3 hours. While this is most probably due to depletion of O_2 and accumulation of CO_2, the possibility exists that other factors such as depletion of respiratory substrate or even production of inhibiting substances (27) may have been partly responsible. To avoid complications arising from differential effects of these factors upon the two processes to be studied and possibly correlated, no measurements of respiration were made after periods longer than 3 hours.

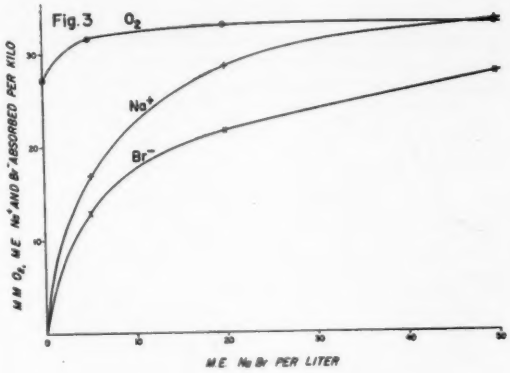
Dependence of Salt Respiration upon Concentration: Three experiments were devoted to an investigation of the relationship between respiration, absorption and concentration of various salts in the external solution. The data obtained are shown in figures 2, 3 and 4. The experimental period was 3 hours and the root to solution ratio, 5 gm/l.

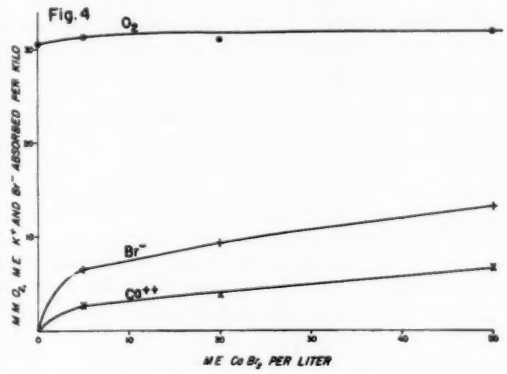
The data depicted in figures 2 and 3 indicate that the effect of salt upon respiration becomes maximal at relatively low concentrations, no increase in respiration resulting from addition of salt above 0.01 *N*. Above this level, however, absorption of both cation and anion continues to be a function of concentration. This is in agreement with results obtained by Robertson and Wilkins (19) working with carrot discs.

The theory of Lundegårdh described in the introduction, according to which electrons carried outward by the cytochrome system are exchanged for anions when oxidation of cytochrome occurs, postulates a maximum value of 4 for the ratio, anions accumulated/O_2 consumed in salt respiration. When the accumulation of inorganic anions from the medium is proceeding at the maximum possible rate, i.e., when the oxidized cytochrome molecules are "saturated" with such ions and are not transporting any internally produced anions, the value of this ratio must be maximal at 4. This postulate is derived simply from the fact that 4 electrons are accepted in the reduction of each molecule of oxygen utilized in the reoxidation of cytochrome oxidase.

It is evident in the data presented in figures 2, 3 and 4 that constancy of the ratio was not shown by this material. The ratio tended to increase with con-







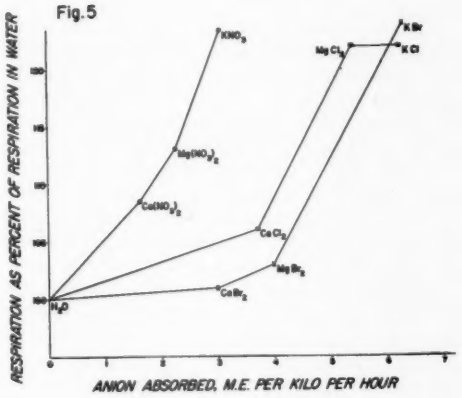


FIG. 2. Respiration and absorption vs conc of KBr.
FIG. 3. Respiration and absorption vs conc of NaBr.
FIG. 4. Respiration and absorption vs conc of $CaBr_2$.
FIG. 5. Respiration vs anion absorption in 0.005 *N* solutions of nitrates, chlorides and bromides.

centration of salt in the medium. Values in excess of the postulated maximum were obtained with all three salts, ranging from 4.3 to 7.9 in KBr, from 2.8 to 4.6 in NaBr and from 9.3 to 18.7 in $CaBr_2$.

The results obtained with $CaBr_2$ are especially striking since only a very slight and possibly not significant increase in respiration was obtained although absorption of bromide from this salt at 0.005 *N* is approximately half that occurring in a solution of KBr at the same concentration. The very small increase in respiration indicated may possibly be due to the presence of small amounts of alkali metals as impurities or may be wholly fortuitous. Repeated trials with $CaBr_2$ failed to show any signficant stimulation by this salt.

To be consistent with the Lundegårdh-Robertson hypothesis the passivity of $CaBr_2$ could not be explained as due to a specific depressant effect of Ca^{++} upon the activity of the cytochrome system since, in this case, bromide absorption would, of course, be suppressed along with the salt respiration. There remains, however, the possibility that Ca^{++} exerts such an effect upon some process associated with the non-cytochrome mediated basal respiration. Conceivably, in the latter case, a bromide induced stimulation of the cytochrome system might be masked when total respiration in $CaBr_2$ is measured. In an attempt to clarify this point the following experiment was carried out. Respiration and bromide absorption were determined in solutions of KBr, $CaSO_4$, KBr + $CaSO_4$ and in water in order to obtain a reference value. Measurements of respiration and absorption in this experiment were made at ½ and 1½ hours after the excised roots were placed into bottles. In view of the linearity of the time curve in this interval the differences between O_2 and Br^- absorbed measured at ½ hour and at 1½ hours represent rates of respiration and absorption. A root solution ratio of 3 gm/l was used. The results are shown in table I.

The lack of any depressant effect of $CaSO_4$ upon total respiration makes it unlikely that Ca^{++}, at this level at least, exerts any such effect upon the basal

TABLE I

EFFECT OF $CaSO_4$ UPON SALT RESPIRATION AND ABSORPTION IN KBr SOLUTION

MEDIUM	RESP RATE	SALT RESP RATE	Br^- ABSORP	INCREASE IN SALT RESP	INCREASE IN Br^- ABSORP
	millimoles O_2/kg fresh wt × hr		*meq/kg fresh wt × hr*	*%*	*%*
H_2O	10.1	..	..	...	...
$CaSO_4$	10.1	0	..	...	...
KBr, 0.005 *N*	11.7	1.6	6.5	...	...
KBr + $CaSO_4$, 0.001 *N* ..	12.3	2.2	7.9	37.4	21.6

respiration; the salt respiration due to $SO_4^=$ would presumably be negligible since it is only very slowly absorbed by this material.

The stimulation of bromide absorption by Ca^{++} in certain concentration ranges has been known for some time; it is sometimes called the "Viets effect" being named for its discoverer (14, 26). The mechanism of this effect is still a matter of speculation. It is seen in table I that this stimulation of bromide absorption by $CaSO_4$ was accompanied by a corresponding increase in the salt respiration. A specific depressant effect of Ca^{++} upon either the basal or salt respiration was then not found. The results are consistent with the supposition that absorption of bromide entails no stimulation of respiration, the effect noted with KBr and NaBr being due perhaps to the cations absorbed; the effect of Ca^{++} upon absorption of K^+ is similar to that of Br^- (26).

ROLE OF CATIONS IN SALT RESPIRATION: It was earlier noted that the hypothesis orignated by Lundegårdh places special emphasis upon the role of anions in the production of salt respiration. According to this view cations move into the tissue by a process of passive exchange for hydrogen ions and hence are assigned no vital part in the observed stimulation. The hypothesis further suggests that all monovalent inorganic anions are of the same effectiveness; e.g., absorption of a given amount of bromide should be accompanied by the same respiratory response as would the absorption of a like amount of nitrate or chloride.

However, the results of the experiments described in the preceding section suggest that certain cations, either in the absorption process or because of some part they play in metabolism after absorption, may affect the respiration rate.

To further investigate the possible role of cations in the stimulation of respiration, experiments were carried out in which respiration and absorption were measured in solutions of 1) K, Mg and Ca nitrate, 2) K, Mg and Ca bromide and 3) K, Mg and Ca chloride.

The effect of slowly absorbed cations in reducing the absorption rates of their associated anions in single salt solutions is well known. It was to be expected therefore that the amount of nitrate, bromide or chloride absorbed from solutions of the same anionic concentration would depend upon the cation associated.

If the cation were inactive in the stimulation of respiration, a plot of anion absorbed (NO_3^-, Br^- or Cl^-) versus respiration should result in a straight line regardless of the nature of the cation associated with a given value of anion absorption.

The results of these experiments are shown in figure 5. The plot obtained for nitrate salts was approximately linear indicating possibly that in the case of these the principal causative agent in the respiratory stimulation is the anion absorbed. However, some stimulation due to potassium is suggested.

The reduction of nitrate to other forms of nitrogen in the plant might be expected to stimulate respiration whether or not such stimulation is attendant upon the absorption process per se. Nitrate then is perhaps a particularly unsuitable anion for studies of this kind. Hamner (5) reports that in intact tomato plants, the reduction of NO_3^- and the appearance of NO_2^- is accompanied by a parallel depletion of stored carbohydrate and by striking increases in the respiratory rate amounting to 100 to 300 %. The mechanism of nitrate reduction in plants is not completely known. Nitrite is presumably the first intermediate in the stepwise reduction to amino nitrogen since this appears very rapidly in the tissues of plants supplied with nitrate (5). Stimulation of respiration by nitrate may be related to the utilization of alpha keto acids by some other intermediate of the reduction process (possibly hydroxylamine) in the eventual formation of amino acids.

The data obtained in pretreatment experiments to be described subsequently indicate significant declines in the nitrate content of these roots when they are allowed to stand in distilled water. In one such experiment, roots with an intial nitrate content of 4.21 meq/kg were found to have 3.00 meq/kg after 1½ hours in water while after 3½ hours this had declined to 1.68 meq/kg. While these changes are probably due to the reduction of absorbed nitrate, the possibility remains that nitrate may have "leaked" from the tissue. The nitrate curve of figure 5 in any case is doubtful since part of the nitrate absorbed was undoubtedly reduced or was lost to the medium after absorption. The extent of the discrepancy between the nitrate absorbed and that found in the roots upon analysis is unknown.

In the case of bromide salts a vastly different picture is revealed. Bromides of calcium and magnesium alike produce little stimulation whereas KBr is again very effective. This again suggests that absorption of bromide does not affect the respiratory rate.

The experiments with chloride salts produced unexpected results in that the stimulation caused by $MgCl_2$ equaled that of KCl although the amount of chloride ion absorbed from its magnesium salt was about 25 % less. This was surprising in view of the almost negligible response obtained with $MgBr_2$. The experiment was repeated with similar results.

DEPENDENCE OF SALT RESPIRATION UPON THE ACCUMULATION PROCESS: In view of the results obtained in the previous experiments which suggest that absorption of certain ions by this material is not accompanied by a rise in the respiratory rate, the question arises whether the salt stimulation observed with many but not all salts occurs as a result of the operation of the absorption mechanism or is due to specific effects of ions upon metabolism after absorption has occurred.

An attempt to answer this question was made by pretreating the excised roots in various solutions and subsequently measuring their respiration in distilled water. Pretreatment was carried out in aerated solutions and in distilled water.

In two experiments (fig 6) the roots were pretreated for 2 hours in 0.005 *N* and 0.010 *N* solutions of KNO_3 and $CaBr_2$, respectively. After pretreatment they were washed and transferred quantitatively to the closed bottles containing distilled water. Respiration was determined in the one-hour interval of ½ to 1½ hours after start. Analyses were made for nitrate and bromide, respectively, and the mean content of these ions during the experimental period calculated as ½ the sum of the contents at the beginning and end of the hour.

It is evident from the data presented in figure 6 that the level of nitrate in the tissue has a decided effect upon respiration in distilled water when presumably the absorption process is inoperative. Robertson and Thorn (16) working with carrot discs which were exposed successively to dilute KCl solutions and distilled water found that a period of 20 hours was required to overcome the effects of the absorbed salt when the electrolyte was replaced by distilled water. Their results are then in agreement with those obtained here.

The data of figure 6 show again the impotency of $CaBr_2$ to affect respiration although considerable bromide is absorbed.

In a third experiment of this type the pretreatment solutions consisted of water, 0.01 *N* KCl and 0.01 *N* $MgCl_2$. Again a stimulated respiration was observed when roots pretreated in these solutions were placed in distilled water after thorough washing. The data obtained are shown in table II.

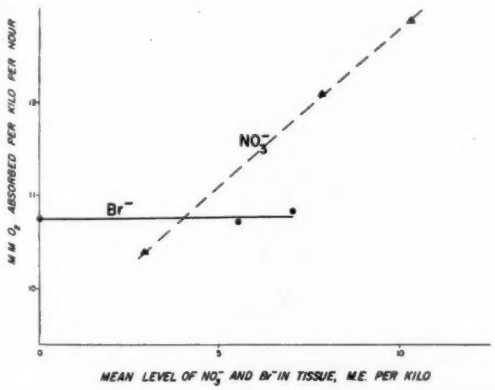


FIG. 6. Respiration in distilled H_2O after pretreatment in $CaBr_2$ and KNO_3 solutions.

TABLE II

EFFECT OF PRETREATMENT UPON RESPIRATION IN H_2O

PRETREATMENT	SUBSEQUENT RESP RATE IN H_2O	MEAN LEVEL OF Cl^-	INCREASE IN RESP RATE DUE TO PRETREATMENT
	millimoles O_2/kg fresh wt × hr	*meq/kg fresh wt × hr*	*%*
None	10.1	2.9	...
Dist H_2O	10.6	2.8	...
KCl, 0.01 *N*	13.2	17.9	24.6
$MgCl_2$, 0.01 *N* ..	13.8	11.9	30.1

These data are consistent with the supposition that an enhanced respiration rate is associated with an increased concentration of certain ions in the tissue rather than with the operation of the accumulation mechanism. However, it must be admitted that other interpretations are possible. While the data of Robertson and Thorn (16) indicate that very little salt is lost from the tissue when it is transferred to distilled water after accumulating KCl, the retention of salt may be ascribed to its continuous loss and reabsorption. The reabsorption process may be efficient enough that the bulk of the medium shows no measurable increase in salt concentration. The concentration of salt in the immediate vicinity of the root is unknown. The data of Robertson and Turner (17) indicating that no loss of salt already accumulated takes place when the accumulation mechanism is poisoned by cyanide are manifestly in conflict with this interpretation.

EFFECT OF VARIOUS SALTS UPON RESPIRATION: During the course of this study respiration was measured in a rather wide variety of media. The results are presented in table III. In each case the concentration of salt used was 0.005 *N*. Respiration was measured for the period between ½ and 1½ hours from start. A control was determined in water in every instance.

It is of particular interest that a considerable stimulation was obtained in 2 cases where presumably no absorption of anions was involved, i.e., in the $KHCO_3$ solution and in the suspension of K bentonite. In the latter case the clay was electrodialyzed for about 10 days, suspended in distilled water and adjusted to pH 7 with KOH.

DISCUSSION

In dealing with such a complex, sensitive, and imperfectly understood system as living plant tissue, it must be admitted that conclusions reached on the basis of measurements of gross effects such as those of salt absorption upon respiration are subject to

TABLE III

EFFECT OF VARIOUS SALTS UPON RESPIRATION

MEDIUM	RESP RATE IN H_2O	RESP RATE IN SALT SOLUTION	INCREASE IN RESP RATE
0.005 N	*millimoles O_2/kg fresh wt × hr*		*%*
KBr	10.1	11.8	16.8
KCl	11.1	13.7	23.4
$KHCO_3$	10.2	12.0	17.6
KNO_3	10.0	12.3	22.6
K_2SO_4	10.3	10.9	5.8
K bentonite	10.6	12.6	18.9
NaBr	10.0	12.1	21.0
$CaBr_2$	10.4	10.4	0
$CaSO_4$	10.3	10.3	0
$Ca(NO_3)_2$	10.0	10.8	8.0
$MgBr_2$	9.9	10.2	3.0
$MgCl_2$	11.1	13.5	21.6
$Mg(NO_3)_2$	10.0	11.3	13.0

varied interpretation. In particular it seems clear that the absorption of any particular ion may entail physical and chemical effects upon aspects of metabolism other than the absorption mechanism per se. For example it is probable that potassium plays an essential part in glycolysis (12) and nitrogen metabolism (4) as well as in the hydration of protoplasm. The effect of potassium upon respiration might then, in the absence of more specific information, be better ascribed to the sum total of its varied effects upon metabolism rather than to any one effect. The same thing might be said for any other ion. For this reason the relation of salt respiration to a general nonspecific effect of inorganic anions upon the cytochrome system postulated by the Lundegårdh hypothesis may be open to question.

In an attempt to show such a simple relationship Robertson and Wilkins (19) have made a rather exhaustive study of the ratio, anions absorbed/O_2 consumed in salt respiration. As indicated earlier, the theoretical maximum value for this ratio is 4. Their results indicate the same want of constancy for the value of the ratio as has been shown here. Very low values (less than one) were found when the medium employed was extremely dilute (6×10^{-4} *N* KCl) and increasing values with more concentrated solutions.

The low efficiency of the salt respiration in very dilute media was, as indicated previously, ascribed to partial satisfaction of the oxidized cytochrome carrier by endogenous anions. This explanation involves a significant departure from the original hypothesis in which the cytochrome system was pictured as being almost inoperative in the absence of inorganic anions. Under this modification the observed stimulations are not simply the result of an inherent necessity for oxidized cytochrome to be electrically satisfied by inorganic rather than organic anions. Rather they are due to some "catalytic" influence exerted by inorganic anions upon the whole cytochrome system, that portion satisfied by organic endogenous anions included. The acceptance of the cytochrome system as the carrier in salt absorption is made more difficult by the finding of values of the ratio in excess of four since this would mean that more anions are being accepted than electrons released—an impossible situation.

The possibility that only certain cations absorbed may be efficient in production of the salt respiration is evident in the data presented in figure 5. The bromide and chloride data are particularly striking. Calcium and magnesium bromides alike have but little effect upon respiration although considerable amounts of bromide are absorbed from these salts. Potassium bromide on the other hand produces a relatively large stimulation. These data suggest that the absorption of Ca^{++}, Mg^{++} and Br^- has little, if any, effect upon respiration. K^+ on the other hand even when accompanied by an ion metabolically inert, such as Br^- causes a marked stimulation. The supposition of a specific effect upon respiration exerted by K^+ is borne out by the effect of K^+ absorbed from a pure bentonite clay suspension and from the bicarbonate, in which cases the possibility of anion effects is presumably eliminated.

Of the ions tested in this study (see table III), K^+, Na^+, NO_3^-, and Cl^- were found to exert stimulating effects upon respiration; Ca^{++}, Mg^{++}, $So_4^=$ and Br^- had little, if any influence. In no case did the presence of salt depress the respiration rate below that obtaining in distilled water. Of the ions whose absorption apparently entails no stimulation, only Br^- is rapidly absorbed by this tissue. Ca^{++}, Mg^{++} and $SO_4^=$ are only very slowly absorbed and the slight effects noted for these ions may be due to this factor. Provisionally it may be postulated that respiration is affected by ions which are both absorbed rapidly and are important in metabolism. In this connection it may be noted that recent work by Broyer et al (1) indicates that chloride may be essential for higher plants.

The results of the pretreatment experiments (fig 6) indicate that stimulations due to salts are not the results of effects exerted upon the absorption mechanism per se. The results shown here are in accord with those obtained by Robertson and Thorn (16) showing stimulation of respiration induced by absorption of salt to be persistent for long periods after removal of the tissue to a salt free medium.

Implicit in the assumption of a direct connection between the salt respiration and the absorption mechanism is the postulate that the plant tissue must do work in order to accumulate salt against a diffusion gradient. The extra energy expenditure is equated with the extra respiration which occurs. However, Robertson (15) has shown that the calculated energy required to effect accumulation from 0.01 *N* KCl is only about 1 % of that freed in the salt respiration accompanying the accumulation. Conversely, the data presented here indicate that ion accumulation may proceed in the absence of measurable salt respiration; considerable amounts of bromide are absorbed from solutions of $CaBr_2$ without any detectable salt respiration for example. These facts, it would seem, cast considerable doubt upon the

assumption that salt respiration represents work done by the plant in accumulating salt.

Overstreet and Jacobson (13) from a consideration of theoretical ion absorption models devised by Rosenberg (21) have shown that if certain appropriate and reasonable assumptions are made regarding the permeability characteristics of the tissue (or at least that part of it through which accumulation occurs), no work may be required for the accumulation of salt against a diffusion gradient other than that work associated with the production and alteration of an ion binding compound at the outside and inside phases respectively; these processes must indeed occur with a decrease in free energy or be linked to other reactions so occurring but the energy released need bear no particular relationship to the amount of work theoretically required for the actual movement of free ions against their diffusion gradient In the Rosenberg models no such actual movement of substances against diffusion gradients is visualized; ions are moved inward only as complexes with metabolically produced binding compounds and always in the direction dictated by the diffusion gradients of these complexes. The possibility of the operation of an ion absorption mechanism similar to one of those devised by Rosenberg makes it unnecessary to postulate on theoretical grounds an increased energy production by tissue when salt absorption occurs. The fact that certain ions, notably Br^-, may be absorbed without appreciable effect upon the respiration rate suggests that although aerobic respiration is essential for salt absorption, this latter process involves particular chemical pathways; these are not necessarily associated with an increase in the overall rate of respiration. The dependence of the accumulation process upon the aerobic phase of carbohydrate degradation further suggests that these particular pathways involve component parts of the tricarboxylic acid cycle. One might speculate that alternate pathways in aerobic respiration involve the production of ion binding compounds from acids of the Krebs cycle. Such a process would involve no unique relationship between the amount of cation or anion absorbed and the stimulation of respiration obtained thereby. Further speculation as to the exact nature of the binding compounds is perhaps unwarranted. However, the response of respiration and absorption to the inhibitor 2,4-dinitrophenol may suggest that the alternate pathways involve phosphorylated compounds (20).

Summary

1. A procedure for the determination of respiration and ion absorption by the same sample of tissue using closed bottles is described.

2. The relationship between concentration of salt in the external solution and the salt stimulation of respiration was investigated. Maximum stimulation was found to occur at concentrations of less than 0.005 *N* when the external solution consisted of KBr and NaBr. Negligible stimulation was obtained with solutions of $CaBr_2$ of concentrations ranging from 0.001 *N* to 0.05 *N*.

3. The values of the ratio, anions absorbed/O_2 consumed in salt respiration, were calculated for various external concentrations of KBr, NaBr, and $CaBr_2$. These values were found to be variable and in some cases to exceed considerably the maximum postulated by the Lundegårdh hypothesis.

4. The possible depressant effect of Ca^{++} upon the ground respiration was investigated by measurement of salt respiration in KBr solutions to which was added $CaSO_4$. No evidence for such an effect was found.

5. The role of cations in the production of salt respiration was investigated. The data indicate that K^+ and Na^+ have a stimulating effect; Ca^{++} and Mg^{++} seem inert in this regard.

6. Salt respiration was found to persist after the roots were pretreated in KNO_3, KCl and $MgCl_2$ solutions and subsequently washed and transferred to distilled water. Similar pretreatment in $CaBr_2$ solutions and in water had no effect upon the respiration rate.

7. A number of experiments were performed to investigate the effects of a variety of salts upon respiration. Stimulation was obtained with $KHCO_3$ solutions and with a potassium bentonite suspension in which cases anion absorption could not have been appreciable. No stimulation was shown by $CaBr_2$ and $CaSO_4$ and only a very small stimulation by K_2SO_4 solutions.

8. The results obtained in this study permit several conclusions to be drawn concerning the nature of salt respiration. These may be listed as follows: a) Stimulation of respiration may be brought about by absorption of either cations or anions or both; it is not uniquely related to the absorption of either. b) The process of absorption need not occur concomitantly with the enhancement of respiration; the presence of certain ions in the tissue affects respiration after absorption has occurred. c) Absorption of certain ions appears to take place in this tissue without stimulating effect upon respiration. d) No fixed relationship is discernible between the amount of any ion absorbed and the amount of respiration occurring in excess of that taking place in distilled water.

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THE HISTOCHEMICAL LOCALIZATION OF PEROXIDASE IN ROOTS AND ITS INDUCTION BY INDOLEACETIC ACID[1]

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Recent evidence (2) has indicated that the peroxidase activity of certain plant tissues rises significantly following the administration of indoleacetic acid (IAA). This phenomenon appears to be related to the induced formation of the IAA-oxidase system (4), of which peroxidase is one component (3). Since peroxidase has recently been shown to be involved in the biosynthesis of lignin (1, 6), the induced formation of this enzyme would appear to have morphogenetic significance.

The purpose of the present paper is to localize in the root those cells and tissues that contain peroxidase, both induced by IAA and non-induced, and to investigate the physiological role of the induced peroxidase in the development of the cell.

Two different histochemical methods were employed. The first involved the use of freshly excised sections and the determination of peroxidase activity

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[2] This work was done when the investigator was a post-doctoral fellow of the National Institutes of Health, National Cancer Institute.

in vivo using conventional biochemical methods on a micro-scale. This approach, while yielding quantitative data, indicates only indirectly which cells participate in the reaction. The second method localizes the enzyme by employing a substrate which yields a colored, insoluble end product. This method, although it indicates the active cells, is only qualitative. Both methods have been used in the present work and their combination offers a quantitative approach at the cellular level to certain problems of cellular growth and differentiation.

Methods

The seeds of *Vicia faba* var. broad Windsor were treated and grown as previously described (5). The seeds were soaked, seed coat removed, and the imbibed embryos placed in moist vermiculite. The growth rate of the primary root is dependent on a large number of factors. Therefore, selection of root tips for experimental work was not based on the age in terms of days after planting but rather on physiological age based on the growth pattern of the root. During the first stages of growth the growing tip of the primary root is relatively thick and somewhat stubby. After the root has attained a length of 2 to 3 cm the growing tip becomes smaller in diameter and then retains this thickness more or less constantly until a period of secondary root formation occurs. Under the growth conditions provided, secondary roots appeared when the primary root was from 8 to 10 cm long. The tips used, then, were from primary roots 3 to 8 cm in length or after initial decrease in diameter and before secondary root formation took place.

Roots were treated with IAA in three ways: 1) the roots of intact seedlings were immersed in an IAA solution in 0.02 M phosphate buffer, pH 6.1; 2) the first 2 cm of the tip was removed and placed in the IAA solution; and 3) the first 3 mm of the root were cut into 200-micron sections and these placed individually in IAA solutions. The duration of IAA treatment was 2 hours. At the end of this time the intact tips (procedures 1 and 2) were washed and cut into 200-micron sections by the method of Jensen (2).

The quantitative determination of peroxidase activity per section was made by placing the 200-micron sections in 0.5 ml of 0.02 M phosphate buffer, pH 4.5. To this was added 0.05 ml 0.1 M H_2O_2 and, at zero time, 0.05 ml of 0.01 M pyrogallol. The reaction was allowed to run 2 minutes. The solution was then rapidly removed from the tissue by a straight, drawn tip pipette and 0.5 ml 1 % HCl-acetone added to the tissue. The absorption of the original solution and in the HCl-acetone was determined at 420 mμ in a Beckman spectrophotometer model DU with a microcuvette attachment. The results are expressed in micromoles purpurogallin produced per section divided by the number of cells in the section.

The localization of peroxidase activity in the sections was achieved by placing the 200-micron section in phosphate buffer at pH 4.5 containing 0.1 M H_2O_2 and 0.1 M guaiacol or benzidine. Activity was manifested as a red color with guaiacol and a blue color with benzidine substrate. Control experiments were performed in which either H_2O_2 or substrate were withheld or in which azide and heating the tissue at 90° C for 5 min were used to inhibit peroxidase activity. Such control sections produced no color over short periods and only little over long periods.

The localization procedures, although imperfect in the sense that the colored end products of peroxidase action are not completely insoluble (8) are adequate for localization of activities in particular cells. They are probably unreliable for intracellular localization, and therefore no attempts have been made in the present work to achieve localization within the cell.

Results

Morphological Analysis: The first 3 mm of the primary root of *Vicia faba* have been previously analyzed in detail (2). The longitudinal diagram of the root in figure 1 indicates the main morphological features of the apex of the root. Six selected 200-micron sections were analyzed for number of cell types or tissues present; volume of each cell type or tissue; cross-sectional area, height, and volume of average cell in each cell type or tissue; number of cells of each cell type or tissue; and total number of cells in each 200-micron section. The complete method of analysis, and results, together with a detailed discussion of their meaning, have been previously presented (5). The number of cells in the various cell types or tissues is presented in figure 3. The decrease in number of cells in the more basal sections of some tissues is the result of increased elongation, decreasing the number of cells in a 200-micron section. The other data from the cell analysis can be summarized by noting that between the general meristem and about 1700 microns behind it the cells undergo rapid division, extensive radial enlargement and limited elongation. Above 1700 microns division and radial enlargement markedly decline while elongation increases rapidly.

Quantitative Determination of Peroxidase Activity: The results of the quantitative determination of peroxidase activity are presented in figure 1. The first fifteen 200-micron sections of the tip were analyzed for peroxidase activity after the root had been treated with IAA by the three different methods. The results are expressed as activity per cell against distance from tip of the section analyzed. Each curve represents at least 4 sets of determinations. The experimental values from these determinations were normally a 10 % range. Occasionally a set would be 20 % higher but with the identical pattern so that when it was superimposed on the other values, without exception the experimental points fall within the 10 % limits. When older roots which had begun formation of secondary roots were inadvertently used it was found they had a different pattern than the 3 to 8 cm roots normally used. It is felt that the roots below 3 cm in length would also show a different pattern. These changes in pattern were not investigated.

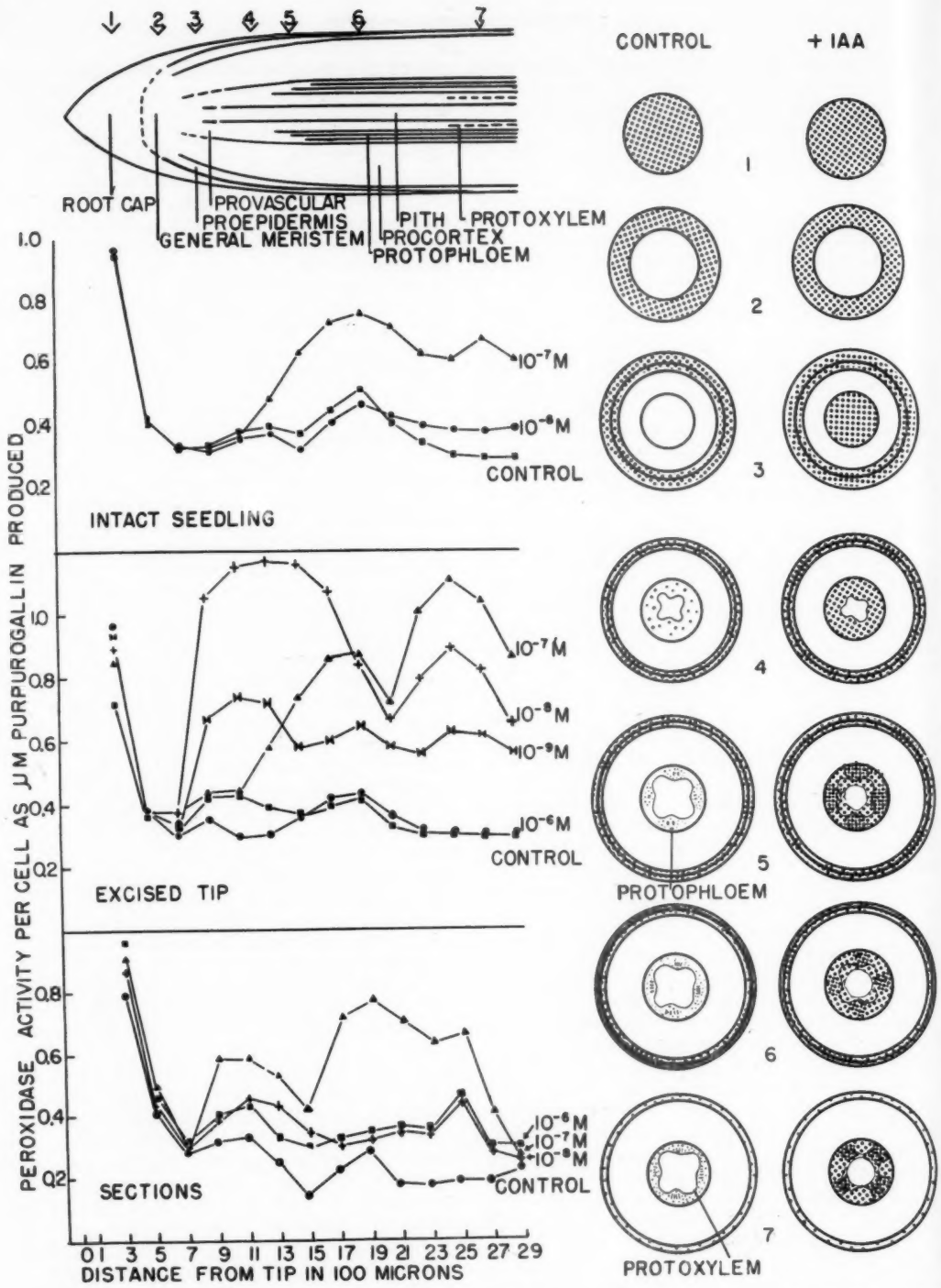


FIG. 1. *Left.* Peroxidase activity per cell as a function of distance from tip of section analyzed, after various treatments of the tips with IAA. See text for examination. Diagram of root on top same scale as graphs. *Right.* Localization of peroxidase in control and IAA 10^{-7} M treated roots. The shaded areas indicate a positive reaction with the intensity of the shading approximating the intensity of the reaction. The number of the sections corresponds to the number on the longitudinal diagram at left.

Considering first the data obtained when excised root tips were treated with IAA, it is clear that the region of the general meristem has little peroxidase activity and is unaffected by IAA. Further, while the cells of the root cap have high peroxidase activity, they similarly show no response to IAA. However, in all other areas of the root the cells show increase in peroxidase over the control after treatment with lower concentrations of IAA.

The control sections basal to the general meristem exhibit two maxima in peroxidase activity. The first corresponds with the appearance of the proepidermis and the provascular tissues, while the second corresponds with the formation of the protophloem. IAA at 10^{-6} M raises the first maximum but does nothing to the second. At 10^{-7} M IAA, the first maximum is increased above the control but not above that elicited by 10^{-6} M IAA, the second maximum is increased approximately 100 % and a third maximum appears. This third maximum corresponds with the appearance of the first forming protoxylem and represents a 200 % increase in activity over the control. Treatment with 10^{-8} M IAA causes a large increase in peroxidase activity directly behind the general meristem that obscures both the first two maxima. This large broad maximum corresponds with the early development of the provascular tissue and the protophloem. The additional maximum corresponds, as at 10^{-7} M, to the developing protoxylem. When 10^{-9} M IAA is used the large broad maximum becomes a double maximum, corresponding to provascular and proepidermis formation and to the development of the protophloem. Finally, the third maximum in the region of the protoxylem formation is present but smaller.

Thus, with changing IAA concentration there is a shift in maximum peroxidase induction from one region of the root to another. This shift can be better seen in figure 2 where a three-dimentional graph has been constructed from the excised tip data of figure 1. For clarity 5 sections are plotted instead of 15 and the drawing of the root indicates the regions in which these sections are located. The maximum in response to IAA treatment shifts from 10^{-8} M at the 900-micron level (area of provascular differentiation) to 10^{-7} M at the 2700-micron level (area of protoxylem differentiation). The region of protophloem development shows a slight maximum at 10^{-8} M IAA but both 10^{-7} and 10^{-8} M IAA elicit high response.

When the data from the sections treated with IAA (lowest graph, fig 1) are examined, a quite different relation of IAA to peroxidase is found. The control sections still have two maxima in peroxidase activity but the magnitude of these are shifted so that now the first is larger than the second. Thus, there is a trend in activity downward from the general meristem. This is interesting in the light of the process of self-induction discussed by Galston (2). When the root is sectioned and its continuity lost, the auxin producing site, which appears to be the general meristem, is detached from the basal areas. These areas are now no longer supplied with native auxin and in 2 hours, as auxin content declines, peroxidase activity decreases.

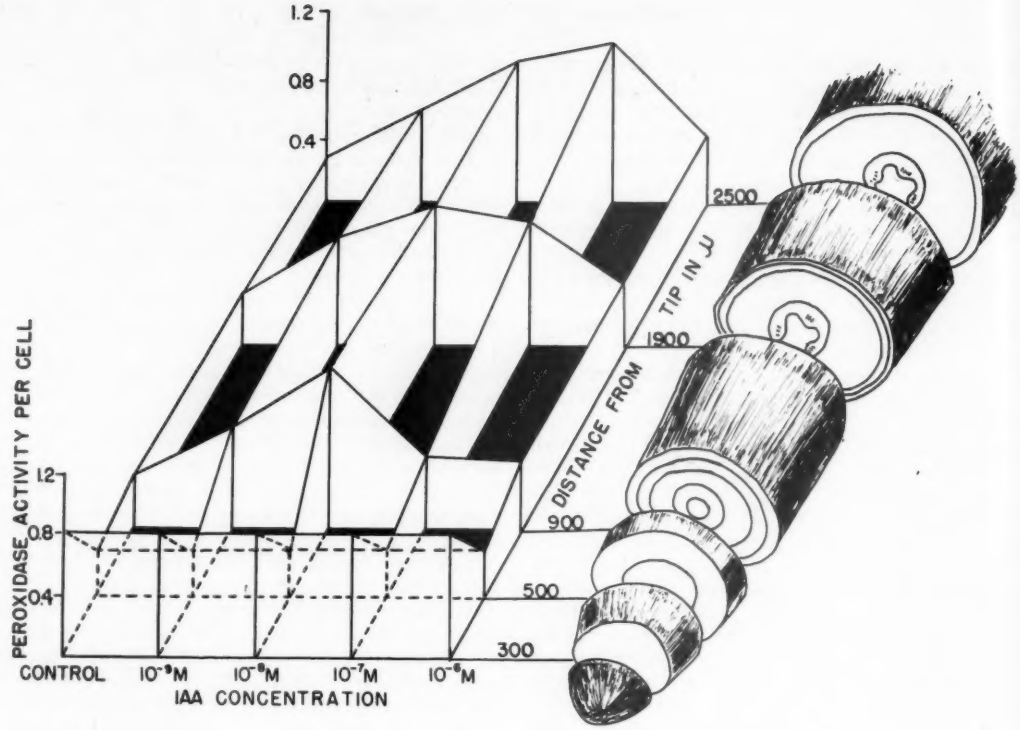
When 10^{-7} and 10^{-8} M IAA is supplied to these sections there is an increase in the first maximum, a general rise in activity that smooths and generally conceals the second, and the appearance of a third maximum. This last maximum corresponds, as with the excised tip, to the beginning of the protoxylem. In all cases, however, with these concentrations of IAA, the amount of induction is less than that produced with the excised roots. Treatment with 10^{-6} M IAA changes the picture drastically. The three maxima are much larger and induction is greatly increased. When the excised tip was treated with 10^{-6} M IAA the peroxidase response was little different from the control, while here the same concentration causes marked induction. One or both of two factors may be involved. First, the native auxin concentration may decrease in the sections and, therefore, higher exogenous IAA concentrations are needed to show the same effect. Second, the cells responding to induction may require substances from other cells to permit induction to occur but, lacking them, can still be induced if the IAA concentration is high enough.

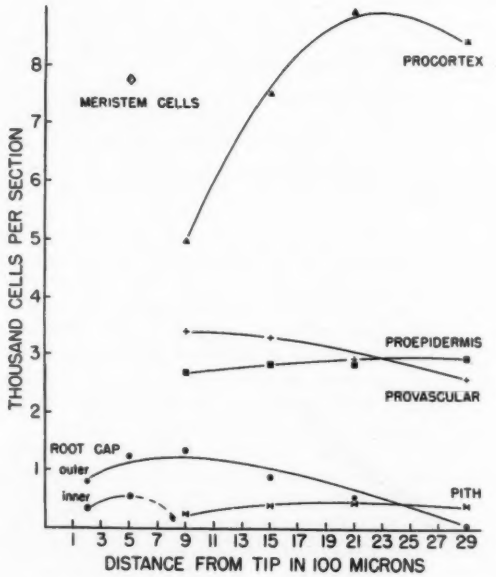
To complete the picture, the roots of seedlings were treated (upper graph, fig 1). In a sense this is the least satisfactory method, as the root is receiving all of its nutrients from the cotyledons and any compound administered constitutes only a small addition. It is therefore not surprising that while the intact root responds at 10^{-7} M IAA, the response is less. The pattern of induction is roughly the same as in the other treatments except that the first maximum is very much lower.

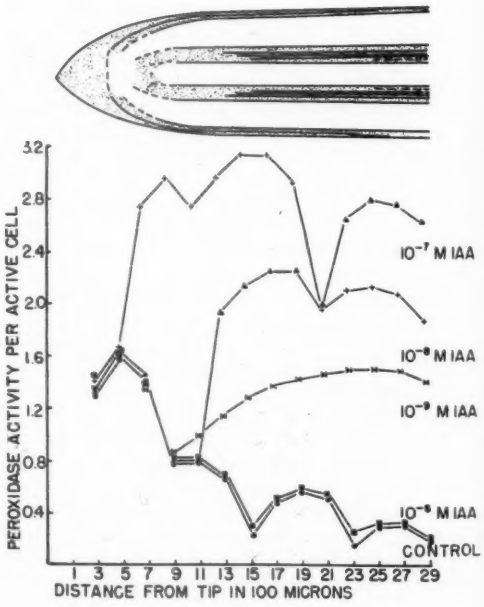
Localization of Peroxidase Activity: The preceding analysis gave strong indications of which cells were active but by indirect rather than direct evidence. By employing localization procedures with guaiacol or benzidine as a substrate, one can visualize the cells that possess peroxidase activity. This has been done and the results are presented as the series of cross section diagrams in figure 1. The control and + IAA roots were treated as in the case of the excised root for quantitative determination up to the time of localization. The IAA concentrations were 10^{-7} and 10^{-8} M.

The results indicate that 1) not all cells at each area are reactive; 2) induction does not cause new tissues to become active but rather increases the activity and number of cells within the tissues that show some activity in the control; 3) those areas of the provascular tissue in the IAA treated roots that will form protophloem and protoxylem are distinguishable by their peroxidase activity before they are morphologically visible in the control.

With or without exogenous IAA the cells of the general meristem, procortex, and pith show no peroxidase activity. This does not completely exclude the possibility that they may have very low activity that is not apparent in these tests. Those cells that show


1.2
0.8
0.4
PEROXIDASE ACTIVITY PER CELL
2500
1900
900
500
300
DISTANCE FROM TIP IN μ
CONTROL
10⁻⁹M
10⁻⁸M
10⁻⁷M
10⁻⁶M
IAA CONCENTRATION


MERISTEM CELLS
PROCORTEX
PROEPIDERMIS
PROVASCULAR
ROOT CAP
outer
inner
PITH
THOUSAND CELLS PER SECTION
DISTANCE FROM TIP IN 100 MICRONS


10⁻⁷ M IAA
10⁻⁸ M IAA
10⁻⁹ M IAA
10⁻⁶ M IAA
CONTROL
PEROXIDASE ACTIVITY PER ACTIVE CELL
DISTANCE FROM TIP IN 100 MICRONS

peroxidase activity compose the root cap, proepidermis, and provascular tissues, including the protophloem and the protoxylem. After IAA treatment the provascular tissues show more intense and extensive activity. The proepidermis may also be more active but not clearly so, as with the provascular tissue. Within the provascular tissue those cells that will compose the protophloem and protoxylem are much more reactive and, in both cases, are identifiable on the basis of their peroxidase activity closer to the tip than it is possible to identify them in the control. The cells that will form the endodermis are clearly delineated by their peroxidase activity approximately 2700 microns from the tip while the endodermis is not distinct morphologically for several more centimeters.

PEROXIDASE ACTIVITY PER ACTIVE CELL: In figure 1 the peroxidase activity is expressed on the basis of the total number of cells in the section. But from the localization work it is clear that not all the tissues in any section are active. Since we already know the number of cells in the different tissues in each section (fig 3), we may calculate peroxidase activity per active cell by dividing the total activity of the section by the number of cells in the active tissues. This has been done for the excised tip data and the results are presented in figure 4. The data now form a series of discontinuous curves rather than a single continuous one. The maxima of the various curves show a remarkable correlation with the appearance of certain cell types. A maximum in activity now appears at 500 microns where the other calculations indicate a minimum. This is a result of the cells of the general meristem being inactive and thus masking the activity of the root cap cells.

The conclusions from figure 4 are: 1) the cells of the root cap, proepidermis, and provascular tissues including the protophloem and protoxylem each have a period of maximum peroxidase activity, and 2) only the cells of the vascular tissues seem capable of induced peroxidase formation.

All the tissues mentioned above have cells with heavy cell walls, but only the vascular tissue, which is capable of induced peroxidase formation, has appreciable quantities of lignin in the mature cell wall. When mature portions of the root of *Vicia faba* are stained for lignin, elements of the phloem and xylem as well as the Casparian strip of the endodermis react.

PEROXIDASE ACTIVITY AND LIGNIN FORMATION: The correlation of the cells that show induced peroxidase formation with the cells that form lignin led to attempts to accomplish lignin synthesis in the sections. Siegel (6) has shown that lignin is biosynthesized by the action of peroxidase–H_2O_2 on hydroxyphenylpropane precursors such as eugenol. The excised root tips were therefore incubated for 2 hours with 10^{-7} M IAA or buffer at pH 6.1 and then cut into 200-micron sections. The sections were individually placed in 0.5 ml, pH 4.5 phosphate buffer that contained 0.1 M eugenol and 0.1 M H_2O_2. After one hour the sections were washed, chlorinated with acidified sodium hypochlorite, washed and placed in sodium sulfite solution. After a few minutes they were washed and either mounted on a slide in dark Karo syrup or dehydrated and mounted in Permount. With this staining procedure lignin should be colored red.

The sections thus treated clearly show that all the cells that have peroxidase activity form a brown compound that is associated with the cell wall. It is believed that this compound is the same as the intermediate in lignin synthesis discussed by Siegel (6). It has the same solubility characteristics as lignin and its formation is inhibited by cyanide and azide. Control tissue from which either eugenol or H_2O_2 was withheld did not produce the compound.

The vascular tissue of the control had a few cells that showed lignin formation, namely elements of the phloem, xylem, and endodermis. The vascular tissue of the IAA treated roots, on the other hand, showed lignin formation in almost all of the cells of the protophloem and protoxylem. The endodermis also showed intense activity.

DISCUSSION

The IAA-induced formation of peroxidase appears to be a possible factor of great importance in cellular differentiation, particularly of vascular tissue. It is significant that the cells respond very early in their development, often before there is any way of identifying them morphologically as different. That the induced peroxidase appears involved in lignin synthesis is of interest as it indicates a physiological role for the enzyme in a process that later manifests itself morphologically.

The observations on lignin formation help explain one of the most common morphological effects of IAA on roots, namely the occurrence of vascularization and lignification closer to the root meristem (7). The sequence of events would appear to be that the cell in the presence of increased IAA forms a system which destroys IAA; part of this system is a peroxidase that is also active in the formation of lignin. Hence, if a peroxide and eugenol type compound are present, lignin may be formed in cells previously exposed to auxin.

Several questions still remain unanswered. First, why is not mature lignin, i.e., the form that will give

FIG. 2 (*top*). Effect of various concentrations of IAA on the peroxidase activity in various regions of the root. The drawing of the root indicates the area of the root from which the sections analyzed were taken.

FIG. 3 (*bottom, left*). The number of cells in the various tissues as a function of distance from tip. The root cap cells, the proepidermis and the provascular cells show peroxidase activity.

FIG. 4 (*bottom, right*). Peroxidase activity *per active cell* as a function of distance from tip of the root of the section. Diagram of root at top is on the same scale and shading indicates those tissues showing peroxidase activity.

the typical lignin staining reaction, formed? It may be that another necessary enzyme is absent in this area of the root or that eugenol is not a completely satisfactory substrate for the reaction. Second, if the root cap and epidermis have the potentiality for lignin production why do they not make it normally? The answer may be the lack of either a satisfactory eugenol-type substrate, or a peroxide, or the presence of inhibitors.

Since the induction of any new peroxidase activity probably involves protein synthesis, it is instructive to examine available data on the locale of protein synthesis in the root. Protein nitrogen per cell has been measured in *Vicia faba* roots (5). When the protein nitrogen pattern is compared with the peroxidase pattern of the excised tip, the rise between 500 and 1100 microns in peroxidase activity at 10^{-8} M IAA closely parallels a rise in protein content per cell. Above 1100 microns, however, the protein nitrogen decreases per cell while the peroxidase activity shows an increase at 1700 and 2500 microns. This latter dissimilarity probably means that the content of protein per cell is a resultant of synthesis and degradation and that even while total protein per cell is declining, protein formation, some of which is peroxidase, is still continuing. The protein pattern does mean, however, that if peroxidase activity were expressed on a protein basis instead of a per cell basis, much of the induction pattern would be lost.

The results illustrate quite clearly the value of a histochemical approach based on a careful morphological study of the organ involved. Neither the quantitative determination of activity of the section nor the localization studies alone offer the information furnished by the combination of both. The application of these same methods to other enzymes and other organs promises to yield valuable data relating enzymatic activities to developmental patterns.

Summary

Peroxidase activity was localized and measured in 200-micron sections of the primary root of *Vicia faba* after treatment with IAA to elucidate the physiological role of the induced peroxidase in the cell. Only the first 3 mm of the root were used as this area had previously been morphologically analyzed in detail.

The cells of the root cap, proepidermis, and vascular tissue including the protophloem and protoxylem show peroxidase activity. However, only the cells of the vascular tissue clearly show the induced formation of peroxidase. The maximum response to IAA shifts from the early provascular tissue at 10^{-8} M to the developing protoxylem at 10^{-7} M.

The cells that contain peroxidase activity are capable of producing a compound when given eugenol and H_2O_2 which appears to be an intermediate in lignin biosynthesis. The vascular tissue that has high induced peroxidase is very active in lignin formation.

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SOME PHYSIOLOGICAL ASPECTS OF BUNT RESISTANCE IN WHEAT[1]

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The very pronounced effect of temperature on the expression of bunt (caused by *Tilletia caries* (DC.) Tul.) in Baart wheat has been reported by Griffith, Zscheile, and Oswald (1). The usual final expression of bunt results in fruiting of the fungus (formation of chlamydospores) in the place normally occupied by the embryo developing into the wheat kernel. Under suitable conditions the resistant variety Baart 38 may permit very limited chlamydospore development (1) in the form of smaller bunt balls, partially bunted kernels or heads, or small number of immature spores hidden within the interior of the normal-appearing kernels.

Since the experimental conditions reported (1) involved certain specific values for light quality and intensity and for the light-period, it is desirable that the relationships of these conditions be studied further in relation to race T-1 of *Tilletia caries*. Optimum values of these conditions for the more nearly complete expression of bunt susceptibility of for maximum differentiation between varieties with respect to bunt resistance, should be determined. These studies

[1] Received April 11, 1955.

report results of several combinations of these environmental conditions.

Methods and Results

Experiment A—Effect of Light-Period in Greenhouse: In the work reported earlier (1) a light-period of 20 hours was used. Since complete susceptibility of Baart was realized in the greenhouse in one experiment (1), it appeared desirable to employ the same conditions of temperature and light during the very early period immediately following germination but to vary the light-period during the remaining much longer time of growth and development to maturity. Similar expanded tests were made on another susceptible variety, Bunyip, and on Baart 38, a resistant strain which resembles Baart except that it carries the M gene (derived from the variety Martin) for bunt resistance (4).

Surface-sterilized seeds were inoculated with dry chlamydospores, planted in pots (1) on January 22, 1952 and germinated at 10° C. For one week after emergence the seedlings were grown at 10° C, irradiated with white incandescent light of 160 fc intensity (provided by one 200-watt clear bulb). All were then moved to the greenhouse on February 8, 1952. Half the pots were grown under natural daylight conditions and the other half were irradiated after sundown with white incandescent light of 150 fc, to make the total light-period of 20 hours correspond with the period used previously in laboratory studies (1).

The light-period provided by the natural daylength varied from 10.5 hours at the start of the experiment to 14 hours at the end in April or 14.8 hours in June. Results are summarized in table I.

Baart and Bunyip produced well-developed vigorous plants under natural daylength, much better than with a 20-hour light-period. The heads were also better developed and many more bunt balls were formed per head. Tillers were well developed on Baart but were without heads. Bunt susceptibility was essentially complete in both Baart and Bunyip under natural daylength. However, with the 20-hour day, although Baart was heavily bunted, Bunyip expressed considerable resistance and produced more tillers and more normal kernels than under natural daylength. In general the 20-hour day produced shorter plants, and more poorly-developed heads; also 26 of 27 Baart plants, which matured first (March 25), had rolled flag leaves, not observed in other groups. In Bunyip both the main stem and tillers were thin, but many tillers had heads. Many Bunyip plants died without producing heads. Baart 38 (resistant) tillered less under a 20-hour day and developed smaller heads and fewer kernels per head. Under natural daylength Baart 38 developed less vigorously than Baart, though some tillers had heads, and the formation of a few blasted heads indicates that the infection had a deleterious effect on this variety, even though final expression of bunt was not realized.

The 20-hour day during such an extended period in the greenhouse appears too long for maximum expression in Bunyip and is probably a borderline condition for maximum difference in bunt expression between Baart and Baart 38.

Experiment B—Combined Laboratory-Field (and Greenhouse) Environment: Attempts were made to subject seedlings to such laboratory conditions for inoculation, germination and early growth that Baart would be 100 % susceptible and Baart 38 completely resistant, even though the field conditions to which the young seedlings were later transplanted might vary from year to year, as is the case in Davis for temperature extremes and ranges as well as for moisture and sunshine conditions. Some plants were transferred to the greenhouse instead of to the field. Martin was included, since it was the source of the M gene in Baart 38.

Table I

Harvest Data and Bunt Incidence for Two Light-Periods

Wheat vars	Plants	Heads	Height (cm) Range	Height (cm) Average	Tillers per plant	Nodes per rachis	Normal kernels per head	Bunt balls per head	% * Bunt
Natural day									
Baart	48	48	25–80	56	0.8	11.6	0.00	12.9	100
Baart 38	48	31	13–75	42	0.5	8.2	1.77	0.0	0 **
Bunyip	49	48	25–86	54	0.4	13.5	0.02	15.0	98
20-hour day									
Baart	32	36	18–43	34	0.9	6.7	0.00	4.0	94 †
Baart 38	43	44	23–56	40	0.1	6.9	0.50	0.0	0
Bunyip	30	39	15–48	30	1.0	7.7	0.20 ††	1.4	47

* A plant was considered bunted if a head or kernel showed any external evidence of chlamydospores (sorus formation).

** Three heads were blasted, possibly a result of infection by Tilletia. Blasted heads consist of a rachis on which no kernel has developed, whereas in bunted heads one or more bunt balls (mass of chlamydospores in place of kernel) are present or at least one kernel has a sorus on its surface.

† Only one well-developed plant escaped and it matured very late. The other non-bunted plant had a blasted head.

†† Only 8 normal kernels developed (on 3 heads).

TABLE II

COMPARISON OF BUNT INCIDENCE IN GREENHOUSE AND FIELD AS INFLUENCED BY LOW TEMPERATURE DURING AND AFTER GERMINATION

WHEAT VARS	GREENHOUSE *		FIELD **	
	NO. OF PLANTS	% BUNT	NO. OF PLANTS	% BUNT
Series 1 † (no cold treatment after emergence)				
Baart	17	100	29	97
Baart 38	22	0	31	0
Series 2 †† (short cold treatment after emergence)				
Baart	24	100	24	100
Baart 38	23	17	26	0

* Planted in two 18-cm pots, 12 seeds each; entire pots kept in greenhouse, avoiding transplanting.

** Planted 5 cm apart in flats; transplanted to field 15 cm apart in rows 60 cm apart, Jan. 3, 1952.

† After emergence, the greenhouse series was placed in the greenhouse on Dec. 22, 1951; the field series was transferred outside the greenhouse until Jan. 3, 1952.

†† After emergence on Dec. 21, 1951, these plants were kept at 10 to 13° C under blue incandescent lamps at 200 fc for 13 days before transfer to greenhouse or transplanting in the field Jan. 3, 1952.

Seeds, dusted with chlamydospores, were planted December 11, 1951, on sterilized soil, covered with vermiculite in flats and pots, and kept at 10° C for 10 days, after which time seedlings had emerged from the vermiculite. Different groups were then treated as described in the footnotes of table II, permitting comparison between plants with and without a cold period at 10 to 13° C after emergence and between plants grown in the greenhouse and in the field. Controls for the field experiment were planted on December 11, 1951 by sowing seed dusted in the usual manner wtih dry chlamydospores. In these controls no bunt was found among 35 Baart 38 plants and of 42 Baart plants, 45 % were bunted. Bunt records are presented in table II.

This experiment demonstrates the effectiveness of the early cold treatment in promoting the maximum difference in subsequent bunt expression between these two (susceptible and resistant) strains in the field. It reaffirms earlier work (1) in which resistance of Baart 38 was decreased to some extent in the greenhouse. The variety Martin in the greenhouse had died down to the ground and new tillers had appeared at the time when the Baart varieties had mature heads. Growth was poor and though they were kept alive until mid-May, the Martin plants did not bloom and produced no heads.

In contrast to the greenhouse plants which had very few tillers the field plants were all vigorous, with many tillers 90 cm high. In the field, every tiller of Baart was completely bunted (except for the one nonbunted plant). Baart 38 had not a single bunted kernel and the average heights of the plants in series 1 and 2 were equal. The Martin plants (field) were shorter in series 2 (height 72 cm) than in series 1 (height 82 cm). They produced fine heads with no evidence of bunt.

Eighteen seeds of Baart 38 in series 1 and 12 seeds in series 2 (greenhouse) showed no evidence of internal chlamydospores on microscopic examination, made by soaking typical seeds in water until soft, crushing on a slide, and staining with cotton blue (3).

Growth data for the greenhouse series are presented in table III.

Baart 38 matured later than Baart in the greenhouse. Bunt readings were taken in mid-February on Baart and in mid-March on Baart 38. The treatment of series 2 retarded development by most of the criteria studied and promoted tillering in Baart 38.

EXPERIMENT C—EFFECT OF SHORT, WARM PERIOD DURING AND AFTER ANTHESIS: While experiment B was in progress we decided to study other combinations of temperature conditions at various periods in the development of the wheat plant. The use of warmer temperatures of the greenhouse during the major portion of the growing period would be conducive to higher fertility and better seed set, as well as more rapid growth. A more detailed study of temperature and duration of cold treatments, combined with different subsequent growth conditions, was undertaken.

To compromise temperature conditions, hoping to still maintain good expression of bunt in the resistant variety, Baart 38, seeds were planted in pots on February 12, 1952, and kept at 12° C. Seedlings emerged on February 20, and grew under blue incandescent lights at 200 fc in the region 380 to 550 $m\mu$ for 7 days with a 20-hour light-period, after which white incandescent lights were used at 420 fc. On April 11 (50 days after emergence), just before heads emerged from their boots, the plants were moved from the 12° C room to the greenhouse under natural daylength and thus raised through the blooming period to maturity at higher temperatures. Noninoculated Baart was planted to note comparative effects of Tilletia infection. Harvest results are reported in table IV, series 1.

Comparison of Inoculated and Noninoculated Baart Plants: The infection of inoculated Baart caused plants to be shorter and heads less thrifty; tillering

TABLE III

GROWTH DATA FOR THE GREENHOUSE GROUP

WHEAT VARS	HT OF PLANTS (CM)	TILLERS PER PLANT	NODES PER RACHIS	NORMAL KERNELS PER HEAD	BUNTED KERNELS PER HEAD
Series 1 (no cold treatment after emergence)					
Baart	42	1.0	9.8	0.0	8.1
Baart 38 ..	54	0.2	8.8	3.6	0.0
Series 2 (short cold treatment after emergence)					
Baart	44	1.0	6.7	0.0	6.3
Baart 38 ..	41	1.4	8.1	0.8	0.4 *

* The 4 plants involved had 1 (plus 1 partially bunted kernel), 1, 2, and 5 bunted kernels.

TABLE IV

EFFECTS OF LOW-TEMPERATURE PERIOD AND LIGHT QUALITY IN EARLY STAGES OF GROWTH ON DEVELOPMENT AND BUNT INCIDENCE IN THE GREENHOUSE

WHEAT VARS	PLANTS	HEADS	HEIGHT (CM) RANGE	HEIGHT (CM) AVERAGE	NODES PER RACHIS	TILLERS PER PLANT	NORMAL KERNELS PER HEAD	BUNT BALLS PER HEAD	% BUNT
Series 1 (50 days at 12° C under incandescent lights; warm greenhouse temperatures during and after anthesis)									
Baart (noninoculated) ..	27	27	43–92	61	12.0	0.0	3.7	...	...
Baart (inoculated)	53	56	36–76	54	10.3	0.13	3.2	7.8	96
Baart 38 (inoculated) ...	34	39	25–79	47	8.1	0.76	1.8	0.67	24
Series 2 (17 days at 12° C under fluorescent lights; warm greenhouse temperatures before, during, and after anthesis)									
Baart (inoculated)	20	37	30–92	67	10.5	0.9	0.0	14.4	100
Baart 38 (inoculated) ...	17	23	38–110	81	13.0	0.9	6.1	0.0	0

was more prevalent and aphids more abundant on inoculated Baart. Noninoculated Baart plants had very plump kernels and a healthy appearance, as did inoculated Baart (8 to 10 kernels on the two nonbunted plants), upon which bunt balls were very plump. Most heads of inoculated Baart did not emerge completely from their boots and aphids were plentiful between the heads and flag leaves. One head was not bunted on a tiller of a plant which had a bunted primary stem. About one-third of the noninoculated Baart plants (not tabulated) grew to a height of 38 cm but were stunted and developed no heads.

Comparison of Baart and Baart 38 Plants: The Baart 38 plants were fairly well developed (6 with blasted heads were not counted as bunted), and were 1 week later than Baart in heading. Of the Baart 38 plants 42 % had either bunted or blasted heads. Many flag leaves were curled and most bunt balls were not plump and large like those on Baart. Eleven of these plants, including 7 bunted plants, were infected with red spider and mildew. Streaks on the leaves suggested infection with wheat mosaic virus to C. A. Suneson. B. R. Houston kindly made several biological assays on some of these plants, but all such tests for mosaic virus were negative. Six Baart 38 plants (not tabulated) did not develop heads. A severe effect of the Tilletia infection is indicated, even though actual evidence of bunt was not as emphatic as when the cold period lasted throughout the life of the plant (1). The time from emergence to harvest was 77 days, little more than one-half the 138 days required (1) when only low temperatures were employed. Bunt relationships between Baart and Baart 38 are similar to those of experiment B, series 2, in which a 13-day cold period under blue incandescent lamps was employed.

EXPERIMENT D—EFFECT OF LONG, WARM PERIOD BEFORE, DURING, AND AFTER ANTHESIS: In this experiment the growth period at low temperature was further decreased. Seeds of Baart and Baart 38 were dusted with chlamydospores, planted in pots on October 23, 1952, and germinated at 12° C. Emergence occurred 10 days later, after which the plants were irradiated with fluorescent light (half from "white," half from "daylight" 40-watt lamps) at 500 fc, 20 hours per day at the same temperature of 12° C. On November 19, 17 days after emergence, the plants were moved to the greenhouse under natural daylength at 27° C. Baart 38 plants had some difficulty with leaf emergence from the tight coleoptile. All plants had a healthy green color except for a yellow coloration of the tip 3 mm of the leaves. On December 10, all plants were moved to a cooler greenhouse. Plants were not jointing and Baart 38 plants were more sturdy than the Baart plants.

On January 30, 1953, the Baart 38 plants were well headed out and taller than the Baart plants. Baart was beginning to head but emergence from the flag leaf was difficult. Greenhouse conditions were not favorable for optimum growth. Plants were harvested on March 30, 148 days after emergence, and the data are presented in table IV, series 2.

Seven of the Baart 38 seeds were examined with cotton blue and no chlamydospores were found in them. These conditions lead to a clear-cut distinction on the resistance basis between these two strains of wheat. Baart produced large bunt balls, whereas Baart 38 produced many normal seeds. Thus a period of 17 days growth at 12° C after emergence, followed by relatively warm greenhouse temperatures, leads to a maximum difference in bunt response between these two wheat varieties. Bunt incidence in Baart 38 was reduced to zero. Expression of resistance in Baart 38 thus appears to be largely a function of the length of time (following emergence) during which the plants grew at low temperatures, although light conditions complicate these comparisons somewhat. The intensity of the fluorescent light was considerably higher (2.5 times) than that of the blue incandescent lights of experiment B, series 2, which produced identical differences in bunt expression in the field (in the greenhouse, where light intensities were lower than in the field, Baart 38 was 17 % bunted). Light intensity may affect expression of resistance in Baart 38 very considerably; higher intensities soon after emergence appear to increase the expression of resistance by Baart 38.

EXPERIMENT E—EFFECT OF DECAPITATION ON BUNT DEVELOPMENT IN TILLERS: According to generally accepted theories (2), the mycelium of Tilletia, after infection of the young wheat seedlings by hyphae from primary and secondary sporidia, penetrates to the growing point rather soon and thereafter grows to maintain its position in this region as the wheat plant develops. When the head is formed, the mycelium is present to infect it while it is still very young. When elongation of internodes occurs the growing point is simply pushed upward, and the parasitic fungus is already established in the tissues of the head, prepared to initiate chlamydospore formation at the appropriate stage soon after anthesis. The mycelium has the habit of extending its area of penetration by abandoning the older cells behind, leaving them empty of protoplasm, which keeps moving forward as new cells are cut off by developing cell walls. To the writer's knowledge, nobody has reported finding mycelial remains throughout the wheat plant all the way from the head in a maturing plant down to the ground level, nor has anyone studied the matter of tiller infection in detail. It is well known that tillers frequently produce bunted heads, but it is not known how long Tilletia remains alive at the base of a plant after elongation of the stem. This seemed interesting to explore.

Plants produced in a series parallel to those of experiment D (planted October 23, 1952) were used in this experiment. The 20 Baart plants raised to maturity had been 100 % bunted and the Baart 38 had no bunt. This demonstrated that conditions of germination and early growth were adequate to insure complete bunt expression in Baart and that all such plants were successfully infected with Tilletia. Four other pots of plants for each variety had been subjected to the same early treatment to promote infection. On January 20, 1953, Baart plants were 60 cm high and Baart 38 plants were 90 cm, the latter being mostly in the boot stage. All were cut off at a distance of 8 cm from the ground (above the second node) to remove the growing point and to encourage tillering at this period, late in the normal life span of wheat.

Two pots (series 1) were permitted to grow such tillers as they would in the greenhouse. The other 2 pots (series 2) were given a cold treatment at 1 to 12° C for 14 days under fluorescent lights (white, 1200 fc, with 20-hour light-period). The temperature was not uniform but it did not exceed 12° C. The plants were then placed in the greenhouse with the others on February 3. On February 27, tillers were 25 to 38 cm high on plants of series 1 but those of series 2 were very slow in developing. All were placed outdoors for the remainder of their growth period. By March 30, when the normal plants were being harvested (exp't. D) the plants of series 1 were heading, with Baart ahead of Baart 38 in development but shorter in height. In series 2, heights were the same but Baart 38 was still in the boot. Some plants of series 2 had died. Bunt counts were made from April 10 to June 10, whenever plants became sufficiently mature to count bunt balls or normal kernels. Results are summarized in table V.

It is clear that Tilletia had remained in a live and potentially virile condition in the extreme lower parts of the Baart wheat stems. The cold treatment given series 2 indicates also that low temperature during beginning of tillering may influence the development of Tilletia, although the numbers of plants involved are small. Among the Baart plants of series 1 there were 2 tillers with no bunt; 1 was a lone tiller, the other had 3 normal kernels but this plant had another bunted tiller, and was counted as a bunted plant. The cold treatment depressed the height of Baart tillers, but doubled their number per plant as well as the number of bunt balls per plant. Ten seeds from Baart 38 plants of series 2 were examined microscopically with cotton blue and no chlamydospores were found.

SUMMARY

Several combinations of conditions involving temperature, light quality and intensity, and light-period were studied in relation to bunt expression in resistant and susceptible wheats.

The 20-hour light-period during extended periods in the greenhouse appears too long for maximum bunt expression in one susceptible variety and may be a borderline condition for maximum difference between two (resistant and susceptible) wheat varieties.

TABLE V

EFFECT OF DECAPITATION ON BUNT DEVELOPMENT IN TILLERS

WHEAT VARS	PLANTS	HEADS	HEIGHT (CM)		TILLERS PER PLANT	NORMAL KERNELS PER HEAD	BUNT BALLS PER HEAD	% BUNT
			RANGE	AVERAGE				
Series 1 (warm greenhouse temperature after decapitation)								
Baart	9	10	30–80	53	1.0	0.33 *	9.3	77
Baart 38	15	17	23–86	61	1.1	3.3	0.0	0
Series 2 (2 weeks low temperature after decapitation)								
Baart	11	21	15–64	41	2.0	0.0	20.0	100
Baart 38	16	Many	.....	..	Many	...	0.0	0

* All kernels were on one head.

Growth conditions were found which shortened the laboratory period of growth at low temperature, permitting longer periods in the greenhouse or field, and which still led to complete bunt expression in Baart and none in Baart 38. They could be modified to result in appreciable bunt expression in Baart 38 (in the greenhouse).

A long cold period (50 days with 20-hour day under incandescent lights) after emergence, followed by a short warm period in the greenhouse, produced 96 % bunt in Baart and 24 % in Baart 38. The infection had severe effects on Baart 38, even though expression of bunt was not as pronounced as when the cold period was longer.

A shorter cold period (17 days, with 20-hour day under fluorescent lights) after emergence, followed by a long warm period in the greenhouse, produced maximum bunt expression in Baart and none in Baart 38.

Tillers produced late in the life span of susceptible wheat may be heavily bunted, especially if given an additional cold treatment following decapitation of the original stem.

The duration of the cold period is very important in determining the extent of bunt expression and degree of differences between resistant and susceptible varieties. Relative intensities of light at different periods following emergence may also be important in this regard.

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STUDIES ON CYTOCHROME REDUCTASE IN HIGHER PLANTS[1,2]

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Enzymes that catalyze the reduction of cytochrome c by the reduced derivatives of pyridine nucleotides apparently are widely distributed in nature. Reductases highly specific for reduced triphosphopyridine nucleotide (TPNH) have ben purified from yeast by Haas et al (9), and from liver by Horecker (10). Edelhoch et al (6) Vernon et al (22), Mahler et al (14), and Mahler and Elowe (15) have made a thorough study of a reduced diphosphopyridine nucleotide (DPNH) specific cytochrome reductase from pig heart. They demonstrated that the pure enzyme was a flavoprotein containing iron. According to the evidence provided by Mahler and co-workers the reaction may be formulated as follows:

$$\text{DPNH} + \text{H}^+ + 2 \text{ oxidized cytochrome c} \longrightarrow \text{DPN} + 2 \text{ reduced cytochrome c} + 2\,\text{H}^+$$

Both flavin and iron apparently are reversibly oxidized and reduced on the enzyme surface. A DPNH specific cytochrome reductase containing flavin-adenine dinucleotide (FAD) has been purified from bacterial sources by Brodie (2). TPNH cytochrome reductases also contain essential flavin nucleotide but no metal requirements for these enzymes have been established (9, 10). Cytochrome reductase activity with either DPNH or TPNH has been observed in Neurospora by Cheng (3).

The presence of cytochrome reductase in higher plants was first reported by Davison (5). She observed that acetone precipitated fractions from Na_2HPO_4 extracts of pea seedlings catalyzed the reduction of cytochrome c by TPNH generated by the isocitric dehydrogenase system, or DPNH produced by the lactic dehydrogenase system. The activity of her preparations was markedly stimulated by FAD or flavin mononucleotide (FMN). Rosenberg and Ducet (18) also demonstrated the presence of cytochrome reductase in higher plants. They reported that the cytoplasmic liquid from soybean leaves catalyzed the reduction of cytochrome c by DPNH or TPNH generated by the systems used by Davison. Chloroplast preparations from the same leaves exhibited little or no cytochrome reductase activity.

There is conclusive evidence that soybean and pea plants contain enzymes that catalyze the reduction of cytochrome c by reduced pyridine nucleotides. There is little detailed information available, however, concerning affinities of substrates, inhibitor characteristics, and distribution of the enzyme in higher plants. Also no information has been reported concerning the purification of this enzyme from higher plant sources.

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[2] Contribution from the Division of Biological Sciences, North Carolina Agricultural Experiment Station, and published with the approval of the Director as paper No. 629.

The primary objectives of the investigations being reported here were to obtain this information.

Materials and Methods

Source of Enzyme: The purified enzyme was prepared from an acetone powder of the primary leaves of 8- to 10-day-old soybean (*Glycine Max* Merr. var. Ogden) seedlings grown as previously described (7). In preparing the acetone powder, one weight of either chilled leaves or roots was homogenized for 1 minute in a Waring Blendor with 10 weights of acetone at −10° C. The liquid was removed by suction and the residue was blended for 1 minute with 3 weights (with reference to original weight of leaves) of n-butanol (14) at −10° C. This was removed by suction and the residue was then blended for 1 minute with 5 weights of acetone at −10° C. The powder was collected by filtration and transferred to a suction flask which was evacuated at 0° C for 2 hours. The dry powder was stored at −15° C in an evacuated flask or desiccator containing anhydrous calcium sulfate.

Other enzyme preparations used in the various experiments were 0.03 M Na_2HPO_4 extracts of roots or leaves prepared according to Davison (5) and homogenates prepared by grinding in a Ten Brock homogenizer for 3 minutes at 0 to 4° C, 1 weight of tissue with 9 weights of cold 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.4.

Other Materials: DPNH, TPN, FAD and FMN of approximately 90, 85, 30 and 100 % purity respectively were obtained from the Sigma Chemical Company, St. Louis, Missouri. TPN was enzymatically reduced to TPNH by the isocitric dehydrogenase system as previously described (7). The concentrations of DPNH and TPNH solutions were determined with a Beckman DU spectrophotometer by use of the extinction coefficient of 6.24×10^6 $cm^2 \times moles^{-1}$ at 340 $m\mu$ (11). The concentration of FAD solutions was also determined spectrophotometrically by use of the extinction coefficient of 1.13×10^7 $cm^2 \times moles^{-1}$ at 450 $m\mu$ (19). Standard solutions of FMN were prepared from weight. Cytochrome c obtained from the Sigma Chemical Company was assayed for oxidized cytochrome c by use of the difference in extinction coefficients of the oxidized and reduced forms at 550 $m\mu$ as described by Horecker and Heppel (12). Other chemicals used were reagent grade obtained from commercial sources.

Standard Assay Procedure: The method for determination of enzyme activity was a modification of that used by Mahler et al (14). The experimental cuvette contained the following constituents in micromoles: 50 Tris buffer pH 7.4; 0.044 oxidized cytochrome c; 0.013 FMN; and 0.20 DPNH. Enzyme was added (volume depended on activity) and the volume adjusted to 1 ml with water. The reaction was started by addition of DPNH and the optical density of cytochrome c at 550 $m\mu$ was followed with a Beckman DU spectrophotometer. It was necessary to add methylene blue to the blank cuvette in order to obtain readings in the lower and most accurate part of the optical density scale. The slit width was adjusted to 0.06 mm. Using this procedure the rate of reduction of cytochrome c proved to be approximately linear with time for the initial two minutes when 0.14 or less units of enzyme were used.

The unit of enzyme activity is defined as an optical density change of 1.00 per minute in the standard assay. This is the unit used by Mahler et al (14). All enzyme activity measurements were based on the increase in optical density between 15 and 75 seconds after the reaction was started. This was corrected for the change without enzyme. Specific activity is the units of activity per mg of protein. The concentration of protein in cell free extracts was determined by use of Folin's reagent (13) and that in homogenates by total nitrogen analysis (23). The molar concentration of oxidized cytochrome c at the beginning and end of the assays could be calculated using the difference in molar extinction coefficients of oxidized and reduced cytochrome c at 550 $m\mu$ which is 1.96×10^4 (12). Other measurements necessary for this calculation were the optical density of the cytochrome c in the assay cuvette after complete reduction with $Na_2S_2O_4$ and the optical density of the reaction mixture at the beginning and end of the assay period. Optical density increase due to cytochrome c reduction during the assay may be converted into increase in molar concentration of reduced cytochrome c by simply multiplying by the factor 5.35×10^{-5} (21).

Purification of Leaf Enzyme: A crude extract (fraction I) of leaf acetone powder was prepared by stirring for 5 minutes at 0 to 4° C 3 gm of powder with 90 ml of 0.1 M K_2HPO_4 with a subsequent centrifugation at 0 to 2° C at a force of $20{,}000 \times g$ for 15 minutes. The crude extract which was yellow in color was purified by use of $(NH_4)_2SO_4$ precipitation and calcium phosphate gel adsorption. All processes in

Table I

Purification of Cytochrome Reductase from Soybean Leaves

	Fraction	Vol	Total activity	Recovery	Protein	Specific activity
		ml	*units*	*%*	*mg/ml*	*units/mg protein*
I	Crude acetone powder extract ..	80	664.0	...	7.2	1.2
II	30 to 50 % $(NH_4)_2SO_4$ ppt	16	180.0	27.1	8.6	1.3
III	First gel eluate	3	29.0	4.4	1.0	9.7
IV	Second gel eluate	3	26.0	3.9	0.6	14.4

the procedure were carried out at 0 to 4° C and all centrifugations were at a force of approximately 20,000 × g. The essential data of a typical purification experiment are given in table I and are described in detail below.

Fourteen gm of solid $(NH_4)_2SO_4$ were added slowly with constant stirring to 80 ml of crude extract and the solution allowed to stand for 10 minutes. The precipitate was collected by centrifugation for 10 minutes and discarded. Ten gm of $(NH_4)_2SO_4$ were added to the supernatant liquid, and allowed to stand for 10 minutes; then the precipitate was collected by centrifugation (10 minutes) and dissolved in 16 ml of 0.05 M Tris buffer at pH 7.4 (fraction II). Calcium phosphate gel (3.0 ml contained 10 mg dry weight per ml) was added to 15 ml of fraction II and allowed to stand for 15 minutes. The gel was collected by centrifugation for 1 minute, mixed with 3.0 ml of 10 % $(NH_4)_2SO_4$ at pH 8.0, and the gel collected by centrifugation for 1 minute. The gel was mixed with 3 ml of 0.01 M $Na_4P_2O_7$ at pH 7.4, allowed to stand for 10 minutes and the supernatant collected by centrifugation for 2 minutes (referred to as fraction III in table I). The gel was then mixed with 3 ml of 0.1 M $Na_4P_2O_7$ at pH 7.4 and after standing for 10 minutes the supernatant was collected by centrifugation for 2 minutes and is referred to as fraction IV. The specific activity of fraction IV was approximately 12 times that of the crude extract. The slightly yellow enzyme could be stored for one week at − 15° C with an approximate decrease in activity of 50 %.

In each purification experiment it was found necessary to test all fractions for their activities since different acetone powder preparations could not be fractionated by identical procedures. This variation seemed to be associated with the completeness of removal of chlorophyll during preparation of the powder. In certain purification experiments it was necessary to collect protein fractions above 50 % saturation with $(NH_4)_2SO_4$ in order to precipitate a major portion of the enzyme. It was also necessary in some experiments to add calcium phosphate gel a second time before an appreciable amount of enzyme was adsorbed. The necessity for these variations in purification procedure was determined by assaying the various fractions for their activities and protein contents.

Experimental Results

Nature of the System: Experimental results reported in table II show that oxidized cytochrome c is enzymatically reduced in presence of either DPNH or TPNH. The relative affinities of these two hydrogen donors is considered in greater detail in another section of this paper. It is of interest to note that all protein fractions which possessed cytochrome reductase activity also showed diaphorase activity.

In regard to the stoichiometry of the reaction, duplicate determinations of the oxidized cytochrome c that was reduced and the concomitant appearance of DPN (method of Racker, 17) in the standard assay procedure showed that 2.17 ± 0.13 μM of reduced cytochrome c were formed for every μM of DPN that appeared. The reaction then is apparently the same as that catalyzed by the pig heart enzyme (14).

Table II

Constituents of Cytochrome Reductase System

Reactants *	O.D. change/min at 550 mμ
Complete	0.092
Without FMN	0.033
FAD instead of FMN	0.088
Without DPNH	0.000
Without DPNH, with TPNH	0.051
Without enzyme	0.016
Without enzyme, with boiled enzyme	0.015
Without enzyme or FMN	0.000
Without enzyme or FMN, with FAD	0.014

* The complete system contained concentrations of reactants indicated in standard assay. 0.0012 μM of FAD was substituted for FMN and 0.20 μM TPNH for DPNH as indicated. The enzyme consisted of 0.092 units of fraction IV.

Both FMN and FAD strongly stimulated the activity of the fraction IV preparation and from various experiments carried out, the concentrations of the flavin nucleotides used were sufficient for maximum activity under conditions of the standard assay. Since the fraction IV enzyme preparation had been purified only 12-fold and therefore may have contained flavoproteins other than cytochrome reductase, no attempt has been made to identify the natural flavin nucleotide. It is interesting to observe that either FMN or FAD without enzyme catalyzed a slow reduction of cytochrome c by reduced pyridine nucleotides. This confirms the previous observations of Singer and Kearney (20). One of these flavin nucleotides is undoubtedly a natural prosthetic group of the enzyme. This seems to emphasize the importance of flavin nucleotide as the active portion of the enzyme.

The addition of a variety of metal salts at different concentrations did not stimulate the activity of undialyzed enzyme. The effects of metals on dialyzed enzyme preparations are being studied at the present time.

pH Optimum: The effect of the pH of the medium on the activity of the enzyme is indicated in figure 1. The maximum activity with the fraction IV preparation was obtained at pH 8.5. In earlier experiments, however, where crude extracts containing particulate matter were used, a broad optimum was observed to extend from pH 7.0 to 8.0. The assay procedure, therefore, was standardized in this range. Evidently the purification procedure altered the physical or chemical state of the enzyme resulting in a shift in the pH optimum.

Substrate Affinities: The final concentration of oxidized cytochrome c required to saturate the enzyme in the presence of an optimum concentration of DPNH and other known cofactors was approximately 4.5×10^{-5} M (fig 2). The concentration necessary for one-half maximal rate was 8×10^{-6} M.

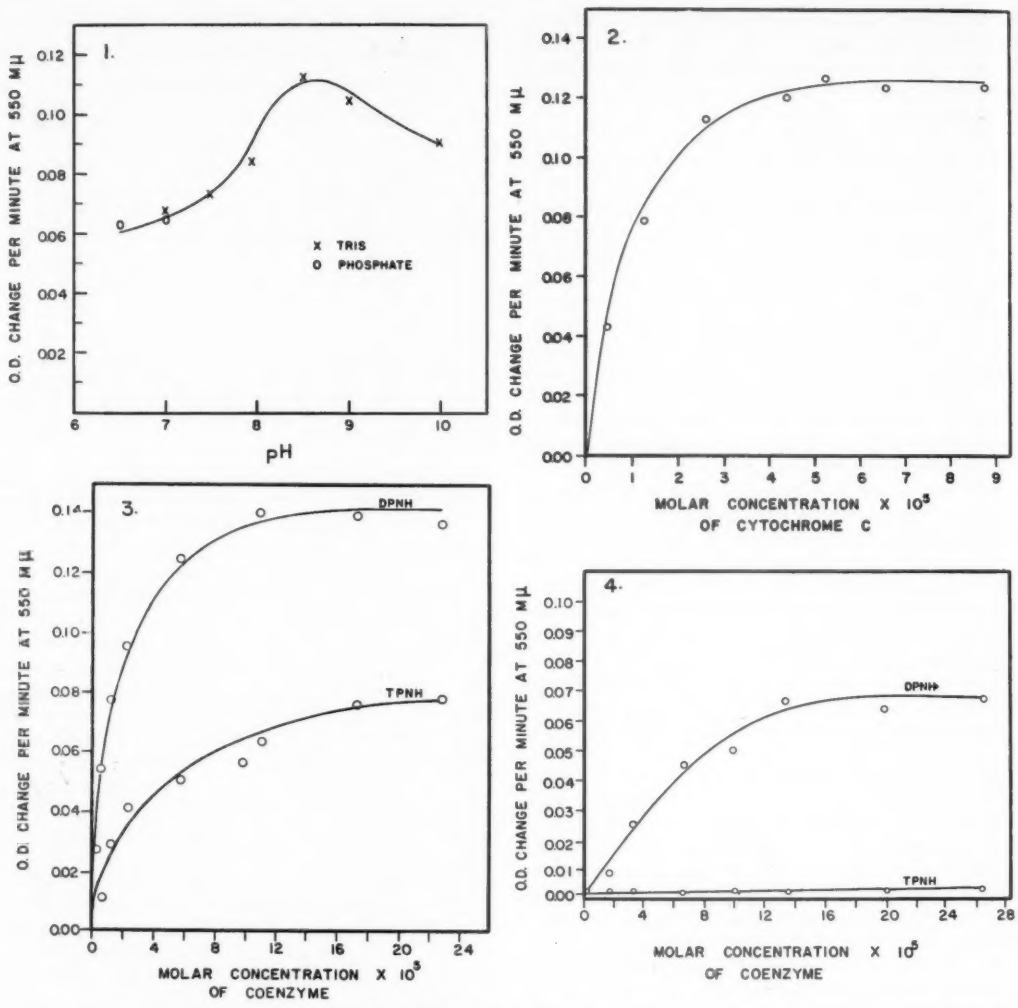

FIG. 1. pH optimum of soybean leaf cytochrome reductase. The standard assay procedure was used with variation in buffers as indicated. The enzyme consisted of 0.068 units of fraction IV.

FIG. 2. Saturation of soybean leaf cytochrome reductase with cytochrome c. The standard assay procedure was used with variation in cytochrome c concentration as indicated. The enzyme consisted of 0.12 units of fraction IV.

FIG. 3. Comparison of DPNH and TPNH as hydrogen donors for soybean leaf cytochrome reductase. The standard assay procedure was used with variation in DPNH and TPNH as indicated. The enzyme used was 0.14 units of fraction IV.

FIG. 4. Comparison of DPNH and TPNH as hydrogen donors for soybean root cytochrome reductase. The standard assay was used with variation in DPNH or TPNH as indicated. 0.068 units of root enzyme containing 45 μg of protein was used which was prepared from a Na_2HPO_4 extract (5) of 9-day-old soybean roots by 35 to 50% $(NH_4)_2SO_4$ precipitation.

The influence of concentration of reduced pyridine nucleotides on the velocity of cytochrome c reduction catalyzed by the leaf enzyme is shown in figure 3. The K_M for DPNH as estimated from the saturation curve is 1×10^{-5} M and that for TPNH is 2.8×10^{-5} M. The maximum velocities of the reaction in presence of the different hydrogen donors indicate that the affinity of the enzyme is considerably greater for DPNH than TPNH (fig 3). The possibility exists that certain constituents in the isocitric dehydrogenase system used to prepare TPNH inhibited cytochrome c reductase activity. To test this possibility

the activity of TPNH prepared in the glucose-6-phosphate dehydrogenase system was compared with that from the isocitric dehydrogenase system and no difference was observed. It seems likely, therefore, that the observed differences in affinity of the enzyme for the two reduced coenzymes is real and not due to an inhibitor in the TPNH preparation.

SEPARATION OF DPNH FROM TPNH REDUCTASE: During the course of these investigations various parts of the soybean plant were examined for cytochrome reductase and it was found that crude Na_2HPO_4 extracts of roots were relatively inactive when TPNH was used as the hydrogen donor. These extracts were very active on the other hand when DPNH was used in the assay (table III). Homogenates or crude acetone powder extracts of roots showed relatively high cytochrome reductase activity with either DPNH or TPNH; however, rates with DPNH ranged around 30 % higher than those with TPNH.

When a protein fraction from the Na_2HPO_4 extract of roots was collected between 35 and 50 % saturation with respect to $(NH_4)_2SO_4$ and examined for activity with the two reduced coenzymes, relatively high rates were observed with DPNH but no activity was found with TPNH. Further tests with this fraction indicated no activity with TPNH over a wide range of concentrations of this reduced coenzyme (fig 4). From these results one may conclude that roots contain two different cytochrome reductases which are separated from each other by centrifugation and $(NH_4)_2SO_4$ precipitation. Another explanation is that roots contain a DPNH specific cytochrome reductase and another enzyme which catalyzes a reaction between TPNH and traces of DPN in the enzyme preparations to produce DPNH and TPN (4). If this assumption is made, then the transhydrogenase is separated from the DPNH cytochrome reductase by the procedure indicated in table III. The characteristics of the DPNH specific enzyme from roots have not been examined in detail.

These experiments raised the question as to the possibility that the various enzyme fractions from leaves were mixtures of cytochrome reductases with different specificities for pyridine nucleotides. Measurements of activities with the four leaf fractions of different purities (table I) were made using both TPNH and DPNH as reductants. The ratios of TPNH to DPNH activity for the fractions were: 0.71, 0.79, 0.72 and 0.57, respectively. The fact that these ratios were not constant during the purification procedure suggests that more than one enzyme was involved. A definite separation of leaf reductases with different coenzyme specificities, however, could not be demonstrated by the procedure that was effective with roots (table III).

INHIBITORS: Samples of enzyme were preincubated for 2 minutes at room temperature with various compounds, then assayed by the standard procedure (table IV). Sodium azide, potassium cyanide, disodiumethylenediaminetetraacetate, *o*-phenanthroline and sodium pyrophosphate, all of which are known to form complexes with metal ions, produced little or no inhibition of activity. Cupric sulfate at concentrations of 10^{-3} and 10^{-4} M inhibited 63 and 55 %, respectively. Manganese sulfate at 10^{-3} M and aluminum chloride at 2×10^{-5} M inhibited 63 and 36 % respectively. *p*-Chloromercuribenzoate which is known to inhibit many enzymes containing essential sulfhydryl groups inhibited the activity 20 % at 10^{-3} M and 35 % at 3×10^{-3} M. The possibility of reversal of *p*-chloromercuribenzoate inhibition by such sulfhydryl compounds as glutathione or cysteine could not be determined because these substances reduced cytochrome c without the presence of a catalyst and therefore interfered with the enzyme assay.

DISTRIBUTION OF ENZYME: Since pyridine nucleotide cytochrome reductase has been observed in peas and soybean plants only (5, 18) it was deemed necessary to examine certain other species for the presence of this system. The various economically important

TABLE III

SEPARATION OF DPNH REDUCTASE FROM TPNH REDUCTASE IN SOYBEAN ROOTS *

PREPARATION **	O.D. CHANGE/MIN AT 550 mμ	
	DPNH	TPNH
	per mg protein	
Homogenate	0.68	0.42
Crude acetone powder extract	1.34	1.08
Crude Na_2HPO_4 extract (3)	2.56	0.33
35 to 50 % $(NH_4)_2SO_4$ ppt of Na_2HPO_4 extract	3.60	0.00

* Standard assay procedure with the following exceptions: TPNH equivalent to DPNH used as indicated; 5 μM of KCN was included in the reactions containing homogenate and crude Na_2HPO_4 extract.

** See text for details.

TABLE IV

INHIBITION OF CYTOCHROME REDUCTASE BY CERTAIN COMPOUNDS *

COMPOUND	FINAL CONC	INHIBITION
	$M \times 10^{-3}$	%
Sodium azide	1	0
Potassium cyanide	1	0
Potassium cyanide	5	0
Disodiumethylenediaminetetraacetate	25	4
o-Phenanthroline	1	17
Sodium pyrophosphate	50	5
Potassium arsenate	10	0
Cupric sulfate	1	83
Cupric sulfate	0.1	55
Manganese sulfate	1	63
Aluminum chloride	2	36
p-Chloromercuribenzoate	1	20
p-Chloromercuribenzoate	3	35

* The standard assay procedure was used with the exception that the compounds listed were incubated with the enzyme (0.075 units of fraction IV) for 2 min before the assay.

TABLE V

OCCURRENCE OF CYTOCHROME REDUCTASE IN YOUNG LEAVES OF CERTAIN PLANT SPECIES *

PLANT SPECIES	AGE	ACTIVITY
	days	*units/mg dry wt*
Soybean, *Glycine Max* Merr.	9	0.42
Lima bean, *Phaseolus limensis* Macf.	8	0.17
Snap bean, *Phaseolus vulgaris* L.	14	0.15
Lespedeza stipulaceae Maxim.	..	0.06
Cotton, *Gossypium hirsutum* L.	25	0.07
Sweet potato, *Ipomoea Batatas* Lam.	..	0.04
Tomato, *Lycopersicum esculentum* L.	22	0.11
Tobacco, *Nicotiana Tabacum* L.	..	0.09
Broccoli, *Brassica oleracea* var. *botrytis* L.	30	0.19
Cucumber, *Cucumis sativis* L.	17	0.41
Peach, *Prunus Persica* Batsch	..	0.02
Barley, *Hordium vulgare* L.	9	0.15
Oat, *Avena sativa* L.	9	0.17
Corn, *Zea Mays* L.	9	0.14

* Standard assay procedure with the exception that FMN was omitted and 0.005 M KCN (final conc) was included. The enzyme was added as homogenates as described in the text.

species listed in table V were included in the survey. In these experiments small aliquots of homogenates (usually 0.02 ml) were assayed for their endogenous capacity to reduce cytochrome c during a 2-minute interval. At the end of the period DPNH was added to start the standard assay. The endogenous rate for the minute just prior to DPNH addition was subtracted from the standard assay rate and expressed per mg dry weight of homogenate. All of the plants tested showed cytochrome reductase activity. The leaves of soybean and cucumber plants appeared to be the best sources of enyme.

DISCUSSION

Cytochrome reductase has been purified approximately 12-fold from an extract prepared from an acetone powder of soybean leaves. When the purification is based on the specific activity of leaf homogenate the purification is approximately 24-fold. The enzyme undoubtedly is still relatively crude in comparison to those from yeast (9), liver (10), and heart (12), which have been isolated in forms approaching pure enzyme. Even though foreign proteins in an enzyme preparation may influence its properties, a comparison of certain characteristics of the soybean leaf reductase with those of analogous enzymes from other sources seems appropriate.

The fact that FMN or FAD strongly stimulated the activity of cytochrome reductase from higher plants, which was originally observed by Davison (5) working with peas and confirmed in these studies using soybean leaves, suggests that the higher plant enzyme is a flavoprotein. It has been demonstrated that the TPNH cytochrome reductases from yeast and liver (9, 10) contained FMN and FAD as prosthetic groups respectively. The DPNH cytochrome reductase from pig heart has been shown by Mahler, Sarkar and Vernon (14) to contain a flavin dinucleotide prosthetic group that was not identical with FAD. The DPNH reductase from bacteria contained FAD as a natural constituent (2). Identification of the natural flavin nucleotide of the soybean leaf reductase must be delayed until further purification of the enzyme has been accomplished.

The pH optimum of the soybean leaf enzyme is at 8.5 which is identical with that of the DPNH reductase from pig heart (14). It is interesting to note that pH activity curves of both enzymes are skewed from the left in a similar manner. The pH optima of the soybean leaf and pig heart enzymes may be compared with those of the TPNH enzyme from liver (10), and DPNH enzyme from bacteria (2) both of which are 8.0. The observation that the pH optimum for cytochrome reductase in crude preparations of fresh leaves was near 7.5 in contrast to a fairly definite optimum of 8.5 for the fraction IV preparation from leaf acetone powder suggests that some physical change in the enzyme took place during the fractionation procedure. Since it can be shown that a large portion of cytochrome reductase of fresh leaf preparations is associated with cellular particulate matter, the shift in pH is probably associated with the breakdown of the particles during purification which released the reductase into true solution.

The specific purification step which was probably effective in releasing the enzyme from particulate matter was the treatment of the acetone powder with n-butanol which removed lipids (16). The properties of a true solution of purified cytochrome reductase then may not necessarily represent the properties of an insoluble reductase which is performing some biological role as a constituent of an organized particle such as a mitochondrion.

An estimation of the Michaelis constant (K_M) of the soybean leaf cytochrome reductase–cytochrome c complex revealed a value of 8×10^{-6} M and the K_M for DPNH was 1×10^{-5} M. Comparable constants found for the DPNH reductase from pig heart muscle were 1.2×10^{-4} and 6.4×10^{-4} for cytochrome c and DPNH respectively. From the concentrations of substrates required to obtain maximum rates it seems apparent that the soybean enzyme has much stronger affinities for both DPNH and cytochrome c than the pig heart enzyme (14). It seems probable that further purification of the leaf enzyme would more likely increase affinities for substrates rather than decrease them. The relatively high affinity of the leaf reductase for cytochrome c and DPNH may be an important characteristic of the leaf enzyme since the evidence suggests that higher plants contain lower concentration of both DPN and cytochrome c than animals or microorganisms (1, 8, 24).

A comparison of the inhibitor characteristics of cytochrome reductase from soybean leaves with those of the pig heart enzyme shows similar effects in regard to heavy metals, but differences with other inhibitors. Both enzymes are strongly inhibited by Cu and Mn

at concentrations of 10^{-3} M (14) but pyrophosphate which caused a 90 % inhibition of 10^{-3} M of the pig heart enzyme resulted in only 5 % inhibition of the soybean leaf reductase when used at the much higher concentration of 5×10^{-2} M. Both enzymes were weakly inhibited by *o*-phenanthroline. The pig heart reductase was far more sensitive to *p*-chloromercuribenzoate than the soybean enzyme. A consideration of the inhibition of the cytochrome reductases from soybean and pig heart sources is of particular interest since Mahler and Elowe (15) have demonstrated that the latter contained iron in a bound form. If it is demonstrated that this metal is a constituent of the plant enzyme, the iron must be bound to the protein in a manner that is not accessible to the various metal chelating agents which have been tested (table IV). It is possible, of course, that the removal of impurities from the plant enzyme may alter its susceptibility to inhibitors.

Summary

Cytochrome c reductase has been partially purified from an acetone powder of soybean leaves and evidence is presented that this or a similar enzyme is present in 15 other economically important higher plant species.

Studies of the characteristics of the purest fraction showed that maximum activity was obtained at pH 8.5 and that DPNH was more effective as a reductant for the system than equivalent amounts of TPNH. The results indicate that the soybean enzyme has a higher affinity for both DPNH and cytochrome c than the cytochrome reductase from pig heart (14). The soybean leaf enzyme was inhibited by Cu, Mn and Al ions and by *p*-chloromercuribenzoate, but was not appreciably influenced by such metal chelating agents as sodium azide, potassium cyanide, ethylenediaminetetraacetate, and sodium pyrophosphate.

It has been demonstrated that soybean roots contain a cytochrome c reductase system that is active with both DPNH and TPNH and that a DPNH specific cytochrome reductase can be separated from crude extracts by centrifugation and $(NH_4)_2SO_4$ precipitation. It was demonstrated also that the ratios of TPNH to DPNH activity of the leaf fractions failed to remain constant throughout the purification procedure, suggesting that more than one enzyme was involved.

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RIBOFLAVIN SYNTHESIS BY EPICOTYLS OF WHITE LUPINE SEEDLINGS GROWN IN STERILE CULTURE[1,2,3]

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In an investigation on the synthesis of riboflavin, thiamin and niacin by excised roots, hypocotyls and epicotyls of white lupine (*Lupinus perennis* L.), grown in culture, it was found that when tryptophan was added to the solution, not only was there an increase in niacin, as expected (4), but there was also an unexpected increase in the riboflavin content of the explants. Further experiments have been made.

Methods and Materials

Epicotyls, dissected from sterile 7-day-old white lupine seedlings, have been grown in culture (5). In early experiments they were placed directly in a modified White's solution (9) to which was added the chemicals under investigation, but in later experiments they were first grown for 3 days in a phosphate buffer of pH 6.2 and 2% sucrose, and then transferred aseptically to the White's solution with the appropriate supplement. The epicotyls were preliminarily cultured in the buffer solution with sucrose with the hope that this might decrease the riboflavin level in the explants, which might later enhance the synthesis in the White's solution. There was no actual decrease in the riboflavin content, but explants grown in the buffer-sugar solution had a much lower B_1 concentration than those grown in the White's solution.

Each culture usually consisted of 70 to 80 flasks, and each flask contained 6 epicotyls. Twenty flasks were devoted to the control and 10 to each concentration of the chemical under investigation. If there was no contamination, all 10 flasks for a concentration were used for the analyses, except when the dry weight was determined, in which case 12 epicotyls were taken for this. The frequency of contamination was not great, and in general, for each riboflavin analysis 36 to 48 epicotyls were used. During the period of culturing, the flasks were shaken on a reciprocal shaker in the dark at a temperature of 26 to 27° C.

Riboflavin has been determined by the fluorescence method of Connor and Straub (2) as modified by the writer for green plants (3). The effect of a variety of chemicals has been studied. Some of these have been auxins, others auxin inhibitors, SH– inhibitors and still others of unknown activity.

Results and Discussion

The first compound investigated and found to give an increase in riboflavin was tryptophan (trypt). This was followed by other indole compounds: indoleacetic, indolepropionic, indolebutyric acids (IAA, IPA, IBA) and indole itself. All of these increased the riboflavin content of the epicotyl (table I) and it was thought for a while that the indole nucleus might be utilized in the synthesis of the isoalloxazine ring. But it was soon found that there was no stoichiometric relation between the concentration of the supplementary compound and the riboflavin concentration.

The next possibility that came to mind was that an increase in growth produced by these auxins might be the cause of the increase in B_1. Naphthaleneacetic, phenylacetic, 2,4-dichlorophenoxyacetic (2,4-D) and 2,4,5-trichlorophenoxyacetic acids (2,4,5-T), which are also growth promoting substances, were tried next, with similar results (table II). Evidently the indole radicle is not a factor; the chlorophenoxyacetic compounds are even more effective than the auxins containing the indole ring.

All of the compounds that had been studied were growth promoters or auxins, and it was considered

Table I

Influence of Indole Compounds on the Riboflavin Content in Epicotyls of White Lupine Seedlings Grown in Sterile Cultures

Expt no.	Compound	Controls		Conc of compound		
				1×10^{-6}	1×10^{-5}	1×10^{-4}
		μgm riboflavin/epicotyl				
507	IAA	0.422	....	0.373	0.467	0.788
508	IAA	0.200	....	0.210	0.270	0.583
515	Indole	0.488	....	0.663	0.726	0.674
541	IAA	0.342	0.343	....	....	0.647
543	IAA	0.410	0.398	....	....	0.816
"	IBA	....	....	....	....	0.533
545	IAA	0.465	0.478	....	....	1.000
"	IBA	....	....	....	....	1.133
"	IPA	....	....	....	....	1.245
"	Indole	....	....	....	....	0.990
"	Trypt	....	....	....	....	0.542

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TABLE II

INFLUENCE OF AUXINS, OTHER THAN INDOLE COMPOUNDS, ON THE RIBOFLAVIN CONTENT IN EXPLANT EPICOTYLS OF WHITE LUPINE SEEDLINGS GROWN IN STERILE CULTURE

EXPT NO.	COMPOUND	CONTROLS		CONC OF COMPOUND				
				1×10^{-6}	1×10^{-5}	3×10^{-5}	1×10^{-4}	3×10^{-4}
				μgm riboflavin/epicotyl				
546	Phenyl ac	.477	.438	.670	.530	....	.571	.753
547	Naphth ac	.563	.455	.567	.677	1.140	1.545	...
550	2,4-D	.367	.380	.532	.987	1.055	....	...
552	2,4-D	.438	.396	...	.768	.875	....	...
"	Phenyl ac	...	...	...	.438		....	...
557	2,4,5-T	.348	.406	.548	1.020	1.200	1.040	.800

Explanations: Phenyl ac—phenylacetic acid; Naphth ac—naphthaleneacetic acid; 2,4-D and 2,4,5-T given in text.

that there might be a relation between the amount of growth of the epicotyls and the vitamin content. The data of many experiments have been examined and some of them presented in table III. The final fresh weight of a culture has been considered as an index of growth. The epicotyls were selected carefully and were of approximately the same weight at the beginning of the experiment, and any difference in the weight of the experimental explants from the controls can be considered as being caused by a change in rate of growth. It should be noted that where two controls were used their final weights were very close. A careful perusal of table III reveals no connection between amount of growth and amount of riboflavin in the epicotyls. In experiment 507 the IAA cultures with high riboflavin had a lower weight than those with a lower riboflavin content, and the same was true with naphthaleneacetic acid and for the higher concentrations of 2,4,5-T, but not for the most effective concentrations of indole and phenylacetic acid, in which increased riboflavin was associated with an increase of growth. In many experiments under consideration dry weight was also determined, but there was no better relation between the riboflavin content and dry weight than between riboflavin and fresh weight.

The antiauxins (6, 7) γ-phenylbutyrate, 2,4-dichloroanisole and 2,3,5-triiodobenzoic acid (TIBA)

TABLE III

COMPARISON OF RIBOFLAVIN CONTENT AND FRESH WT OF EPICOTYLS

EXPT NO.	CHEMICALS USED	CONTROLS		CONC OF CHEMICALS USED				
				1×10^{-6}	1×10^{-5}	3×10^{-5}	1×10^{-4}	3×10^{-4}
507	IAA	.422	...	.375	.467	...	.788	...
		(.285)		(.284)	(.254)		(.233)	
515	Indole	.488	...	.663	.726	...	.674	...
		(.181)		(.211)	(.237)		(.217)	
545	IAA	.465	.478	...	...	...	1.000	...
		(.245)	(.252)				(.239)	
"	IBA	...	...	...	...	...	1.133	...
							(.275)	
"	IPA	...	...	...	...	...	1.245	...
							(.272)	
"	Trypt	...	...	...	...	...	.542	...
							(.253)	
"	Indole	...	...	...	...	...	.990	...
							(.206)	
546	Phenyl ac	.477	.438	.670	.530	...	.571	.753
		(.393)	(.390)	(.440)	(.373)		(.454)	(.465)
547	Naphth ac	.565	.455	.567	.677	1.140	1.545	...
		(.347)	(.344)	(.342)	(.313)	(.362)	(.305)	
550	2,4-D	.367	.380	.532	.987	1.055	...	...
		(.346)	(.352)	(.360)	(.350)	(.400)		
557	2,4,5-T	.348	.406	.548	1.020	1.200	1.040	.800
		(.316)	(.327)	(.319)	(.368)	(.404)	(.287)	(.249)

For each chemical two rows of figures are given: the *lower* with figures in parentheses denotes the fresh wt in gms, and the *upper* gives the riboflavin content in μgm/epicotyl.

TABLE IV

INFLUENCE OF THE ANTIAUXINS γ-PHENYLBUTYRATE, 2,4-DICHLOROANISOLE AND 2,3,5-TRIIODOBENZOIC ACID ON THE RIBOFLAVIN CONTENT OF EPICOTYLS OF WHITE LUPINES

EXPT NO.	CHEMICALS USED	CONTROLS		CONC OF CHEMICALS USED			
				1×10^{-6}	1×10^{-5}	3×10^{-5}	1×10^{-4}
556	TIBA	.393 (.317)	.332 (.268)	.463 (.380)	.484 (.390)	...	.508 (.302)
563	γ-Phenylbutyric ac	.466 (.376)	.466 (.364)	.400 (.323)	.513 (.276)	.516 (.331)	.531 (.309)
565	2,4-Dichloroanisole	.460 (.307)	.440 (.297)	.486 (.307)	.560 (.272)	...	.605 (.183)
566	2,4-Dichloroanisole	.464 (.283)	.470 (.294)	...	...	...	.944 (.207)

The figures in parentheses are the weights in gms/epicotyl and the figures above, the vitamin in μgm/epicotyl.

were also used to see what would happen to the riboflavin content if the growth was decreased below the controls. The results are given in table IV. The two experiments with 2,4-dichloroanisole demonstrate conclusively that an increase in riboflavin is not necessarily associated with an increase in growth. In these experiments the concentration of antiauxin which produced the largest increase in vitamin content significantly decreased growth. The triiodobenzoic and γ-phenylbutyric acids did not produce as striking results but the results were along the same line.

As the work progressed and it became more and more obvious that there was no close relation between the rate of growth and riboflavin content, the thought occurred that maybe the explanation lies not in an increased synthesis of riboflavin but in a sparing action. Riboflavin is a component of several of the respiratory enzymes and it is possible that the increase in the vitamin might be connected with a decrease in respiration. Several sulfhydryl antagonists (1, 8) were tried. The results varied with the compound (table V). Low concentrations of phenylmercuric benzoate and *p*-chloromercuric benzoate produced an increase of about 20 %, but higher concentrations of both compounds caused a decrease in riboflavin concentration. Iodoacetate was quite variable, producing both a decrease and an increase regardless of concentration. Potassium arsenite decreased the amount of vitamin by a concentration of 1×10^{-7} M but an increase occurred at the concentration of 1×10^{-4} M.

The sulfhydryl antagonists did not yield uniform information; two of them decreased the B_1 concentration whereas the other two were rather indifferent, causing both a decrease and an increase. A study of respiration is necessary, but this cannot be done for some time.

SUMMARY

Studies of riboflavin content of epicotyls of white lupines grown in sterile cultures have been made. It has been found that a number of auxins, antiauxins and sulfhydryl antagonists have increased the concentration of the riboflavin in the explants. This is not associated with an increase in growth. From the experiments it is not clear whether there is an actual increase in synthesis or a decrease in use, but it seems more likely to be an increase in synthesis.

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TABLE V

EFFECT OF THE ADDITION TO THE NUTRIENT SOLUTION OF ANTI-SULFHYDRYL COMPOUNDS ON THE RIBOFLAVIN CONTENT OF EPICOTYLS OF WHITE LUPINES

EXPT NO.	CHEMICALS USED	CONTROLS		CONC OF SULFHYDRYL ANTAGONISTS				
				1×10^{-7}	3×10^{-6}	1×10^{-5}	3×10^{-5}	1×10^{-4}
				μgm riboflavin/epicotyl				
555	Iodoacet. ac	.464	.491	...	.553	.451	.424	.566
561	Phenylmercuric benzoate	.420	.427	.503	.367	.292	...	...
562	$KAsO_2$	.388	.397	.298	.365	.382	.481	...
567	*p*-Chloromercuric benzoate	.463	.442	.546	.534	.415	...	.328

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THE INFLUENCE OF RATE OF COOLING AND WINTER COVER ON THE WINTER SURVIVAL OF LADINO CLOVER AND ALFALFA[1,2]

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The loss of perennial forage legumes during the winter season continues to be a major problem. Much is still unknown concerning the winter climates to which plants are exposed and their relationship to damage from low temperatures.

This study deals with winter temperature variations of the air near the surface of the soil and of ladino clover stolons where they are covered with grass or snow and where uncovered. In addition, the injury to alfalfa and ladino clover plants exposed to similar temperature variations was determined in the laboratory. An interpretation of the results from these two studies offers a better understanding of the relationship between fall stubble, surface mulches, and snow cover and winter survival.

METHODS AND PROCEDURE

Ladino clover (*Trifolium repens* L.) and alfalfa (*Medicago sativa* L.) were used as test plants because of their prominence in agriculture. Stolons of ladino clover used in the laboratory studies were selected for uniformity and good vigor. They were taken from the field less than 36 hours before testing and were trimmed to 10 cm in length from the tip-end bud. Each sample consisted of 8 stolons. Three replicates were used in all treatments. Cold hardened plant materials consisted of 2 clones of ladino clover previously selected for differences in cold hardiness plus field run ladino clover from Oregon certified seed.

Alfalfa used in laboratory freezing studies was mostly seedling material of the Buffalo variety sown August 20, 1951. Plants were dug from the field in mid-winter, 1952, washed in cold water and trimmed to root lengths of 10 cm and stem lengths of 2.5 cm. Eight roots made up each sample with 3 replicates for each treatment.

All plant samples were dug from the field, washed, trimmed and immediately inserted into large test tubes. They were then stored in a cool room at 34 to 36° F for 24 hours before applying freezing treatments. After freezing all samples were stored at 36° F for 24 hours before being planted in quartz sand in the greenhouse. The growths made during 21 days were used as indices of the amount of injury.

EXPERIMENT 1: During January, 1951, tubes containing ladino clover stolons from the cold hardy clone (No. 5) and from the less hardy clone (No. 3), were taken from the cool room and placed in an alcohol bath at 36° F. The temperature of the bath was then lowered at a rate of 2° F per hour. Successive samples were later moved from the cool room to the alcohol bath when the temperatures had reached 30, 25, 20, and 15° F. All samples were removed from the alcohol bath when the stolons had reached 10° F except one set which was put in at 36° F and removed when the temperature had reached 8° F. An additional group of samples was not frozen.

EXPERIMENT 2: Buffalo alfalfa plants were removed from the field on January 3, 1952, stored at 34° F and subjected to the same freezing treatments as the ladino clover in experiment 1. Additional samples were exposed to slightly lower temperatures (5° F) in consideration of greater cold hardiness of alfalfa.

EXPERIMENT 3: The principle treatments in experiments 1 and 2 were repeated with alfalfa and ladino clover during February, 1952. Additional temperature ranges were included to test plant tolerances to low temperature more completely. The alcohol bath was cooled at the same 2° F per hour rate from 36° F. Replicate samples of ladino clover and of alfalfa were removed from the cool room at intervals and put into the freezing bath when it had reached 36, 20, 15, and 10° F. Samples were removed when the bath had reached 15, 10, 5, and 0° F.

Samples which were put into the alcohol bath between 36 and 30° F and lowered to the temperature at which the samples were removed at the rate of 2° F per hour are referred to as receiving the "slow freezing" treatment. By comparison, those samples removed from the cool room storage at 36 or 34° F

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and put into the alcohol bath when it was 15, 10 or 5° F are referred to as being "fast frozen."

FIELD TEMPERATURE OBSERVATIONS: Air temperatures were determined during January and February, 1953, 1954 and 1955 using shielded copper constantan thermocouples and a recording potentiometer. In 1954 and 1955 plant temperatures also were determined with very small thermocouples made from No. 30 B. & S. guage wire for insertion into the tissue. The tissues were punctured at an oblique angle with a small needle and the thermocouple inserted. Temperatures were recorded continuously night and day, on some occasions, and during critical periods on others.

RESULTS AND DISCUSSION

LABORATORY FREEZING STUDIES: Fast freezing consistently inflicted more injury to cold-hardened alfalfa plants and ladino clover stolons than did slow freezing. In two other similar experiments not reported the results were the same. Stolons, roots and crowns which were cooled slowly withstood lower temperatures for a longer period of time without dying than tissues cooled rapidly. This comparison was consistent with both clones of ladino clover but the injuries sustained were less with the hardy than with the less hardy clone (table I).

In experiment 2, the injury to over-wintering parts of cold-hardened alfalfa was less when the plants were cooled slowly to 5° F than when cooled rapidly to 10° F. Further, those plants cooled slowly were exposed to freezing temperatures more than 4 times longer than those cooled rapidly (table II, fig 1). Accordingly, the time of exposure to below freezing temperatures did not appear to be critical in these trials.

In experiment 3, the alfalfa roots and crowns exhibited greater tolerance of low temperature than the ladino clover stolons (table III). In general, slow cooling permitted both plants to survive approximately 5° F colder temperatures than did fast cooling. Alfalfa plants cooled rapidly to 5° F were all killed; whereas those cooled slowly to the same temperature made fair recovery. Similarly, ladino stolons cooled rapidly to 10° F were killed and those cooled slowly to the same temperature made good recovery.

TABLE II

RECOVERY GROWTH OF BUFFALO ALFALFA PLANTS DURING 3 WKS IN THE GREENHOUSE FOLLOWING COLD TREATMENTS OF DIFFERENT RATES OF COOLING. ALL SAMPLES WERE STORED OVERNIGHT IN A COLD ROOM AT 34° F BEFORE AND AFTER FREEZING

MINIMUM TEMP REACHED	TIME IN FREEZING BATH	AVERAGE RATE OF COOLING	RECOVERY GROWTH	
			DRY WT PRODUCED	ROOTS ALIVE
°F	hr	°F/hr	gm	%
Not frozen	0	...	1.76	100.0
10	12	2.0	0.67	97.5
10	10	2.4	0.76	100.0
10	7.5	3.2	0.74	91.3
10	5	4.8	0.43	66.2
10	2.5	9.6	0.15	50.0
5	14.5	2.0	0.59	100.0
5	5	5.8	0.05	1.6
5	2.5	11.8	0.08	2.1

The exact interval of time during which any of these plant tissues were below a given temperature was not determined. However, subsequent plant temperature measurements indicated that above 25° F equilibrium to within one degree of the temperature of the alcohol bath was reached within a few minutes after a sample was inserted. With samples put into the alcohol bath at 20° F or lower, plant temperatures dropped rapidly to 27° F and presented approximately a straight line cooling curve thereafter. Accordingly, the principles of fast and slow cooling set forth in this paper remain intact.

During January and February, 1953, small thermocouples were inserted into 4 roots and 4 crown buds of alfalfa and their temperatures recorded at 30-second intervals while the plants were cooling. Samples which were cooled slowly from 36 to 10° F exhibited from 4 to 6° F of undercooling. A freezing point of 26.5 to 27° F was observed. All plants lived. No undercooling was detected in samples cooled rapidly

TABLE I

RECOVERY GROWTH OF LADINO CLOVER STOLONS DURING 21 DAYS IN THE GREENHOUSE FOLLOWING COLD TREATMENTS OF DIFFERENT RATES OF COOLING. ALL SAMPLES WERE STORED OVERNIGHT IN A COLD ROOM AT 36° F BEFORE AND AFTER FREEZING

MINIMUM TEMP REACHED	TIME IN FREEZING BATH	AVERAGE RATE OF COOLING	RECOVERY GROWTH			
			DRY WT PRODUCED		STOLONS ALIVE	
			CLONE 3	CLONE 5	CLONE 3	CLONE 5
°F	hr	°F/hr	gm	gm	%	%
Not frozen	0	...	1.06	0.88	87.5	85.4
10	13	2.0	0.53	.66	61.0	76.9
10	10	2.6	.73	.68	68.7	81.5
10	7.5	3.5	.37	.55	19.0	68.5
10	5	5.2	.05	.31	11.2	61.0
10	2.5	10.4	.00	.08	1.3	17.3
8	14	2.0	.59	.76	54.7	61.7

TABLE III

RECOVERY GROWTH OF LADINO CLOVER AND BUFFALO ALFALFA DURING 3 WKS IN THE GREENHOUSE FOLLOWING COLD TREATMENTS OF DIFFERENT RATES OF COOLING. ALL SAMPLES WERE STORED OVERNIGHT IN A COLD ROOM AT 36° F BEFORE AND AFTER FREEZING

MINIMUM TEMP REACHED	TIME IN FREEZING BATH	AVERAGE RATE OF COOLING	DRY WT OF TOP GROWTH PRODUCED	
			LADINO CLOVER	ALFALFA
°F	hr	°F/hr	gm	gm
Not frozen	0	...	0.98	1.51
15	10.5	2.0	...	0.93
10	13	2.0	0.28	0.54
5	15.5	2.0	0.03	0.45
0	18	2.0	0.00	0.00
15	2.5	8.4	0.13	0.52
10	2.5	10.4	0.00	0.14
5	2.5	12.4	0.00	0.00

to 10° F, and the freezing point was indistinct. All plants died.

These observations were repeated with Grimm alfalfa, African alfalfa, and ladino clover stolons in a similar manner during January, 1954. In these trials, the temperature of the tissues was determined at 15-second intervals and of the cooling bath and air inside the large test tubes every 3 minutes while slow cooling was taking place. Although the temperatures of the African alfalfa and ladino clover tissues were within 0.5° F of those of the surrounding air above 27° F, this relationship at no time existed at temperatures between the apparent freezing point and 10° F. The temperature differences between the air and the tissues were greatest (6 to 7° F) about an hour after the maximum point of undercooling was reached (fig 2). As the temperatures of the less hardy legume

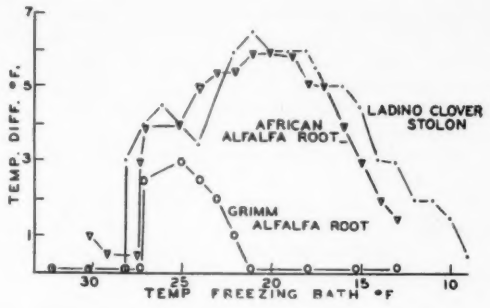

FIG. 2. Temperature differences between plant tissues and the cooling medium (air) under conditions of slow cooling through the freezing point.

tissues again began to drop after undercooling they did not rapidly approximate those of the surrounding air as in the case of the more cold hardy Grimm alfalfa. Rather the tissues cooled slowly and continued several degrees warmer than the air inside the tubes for several hours. This relationship was observed in numerous tests with alfalfa roots and ladino clover stolons. Differences were less under conditions of fast cooling than with slow cooling.

The Grimm variety of alfalfa is well known for its cold hardy characteristics; whereas, African alfalfa has consistently shown less winter-hardiness. The large differences between the curves of these two (fig 2) suggest that the water in the live tissues of these varieties may be in different phases.

Resistance to cold injury of plants has frequently been associated with greater bound water content and osmotic concentration of the cell sap (1). The data obtained in this study tend to support these associations. It is suggested that the apparent freezing point

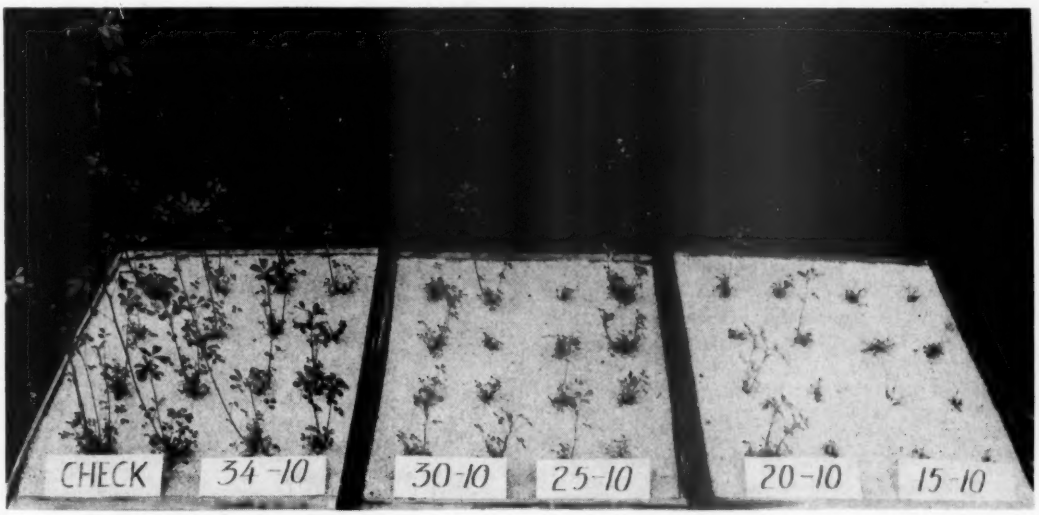

FIG. 1. Recovery growth of alfalfa following freezing treatments of (*left to right*) no freezing, slow and fast cooling. Experiment 2. January, 1952.

of about 27° F observed in these trials is that of adhered water free of most colloidal bonds. Further, the apparent lag in temperature fall of cooled tissue, is a response to heat of crystalization from progressive freezing within the tissue and varies in proportion to the rate of freezing. Each successive amount of water removed from the cell by freezing increases the concentration of the remaining cell sap and increases the bond strengths of the remaining bound water in proportion to the amount of water lost. This concept recognizes no single freezing point but presumes that crystalization under conditions of slow cooling is a continuous process of freezing freed water. Under conditions of rapid cooling water may be unable to move out of the cell at a sufficiently rapid rate to prevent lethal ice formation within the cell (3).

CLIMATIC OBSERVATIONS: Fluctuations in air temperature near the surface of the soil are of greater magnitude and are more rapid than at the standard height of 4.5 feet at which the U. S. Weather Bureau temperatures are obtained. This is accounted for by the ground surface representing the source heated by incoming radiation during the day and, by outgoing radiation, the cold source during the night (2). In winter, the stolon level in the field (0 to 1.5 cm above the soil surface) may be within a cover of grass stubble or snow or may be uncovered. Daily temperature fluctuations of both air and of plant tissues observed at New Brunswick, during January and February, 1953, 1954, and 1955, indicate that stolon temperatures reach greater extremes than air temperatures and temperatures within a cover are far more stable than where no cover exists (fig 3). On clear days air and plant temperature changes at the stolon level were much greater and more rapid where there was no cover than under snow or a grass stubble (fig 4). Further, on frequent occasions, the uncovered stolons cooled from 50 to 10° F in approximately 5 hours, a range and rate approximating that found injurious in laboratory tests. On cloudy days and nights stolon temperatures more closely approximated air temperatures.

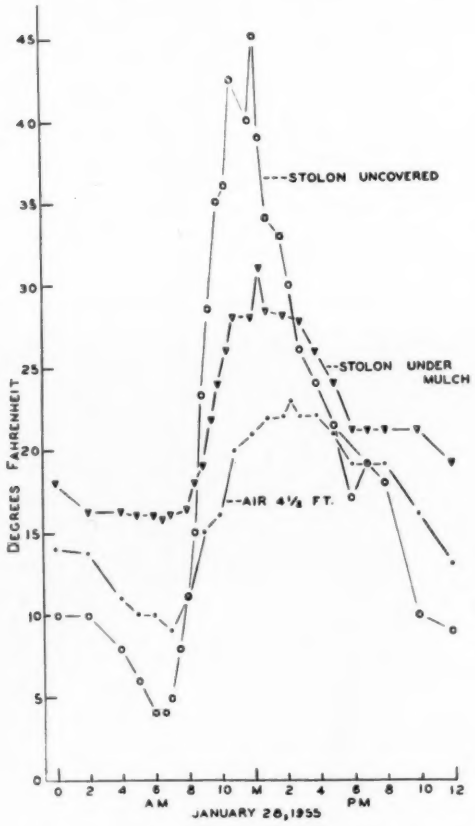

FIG. 3. Characteristic daily temperature fluctuations of air and clover stolons, without snow cover, during mid-winter at New Brunswick, New Jersey. 1955.

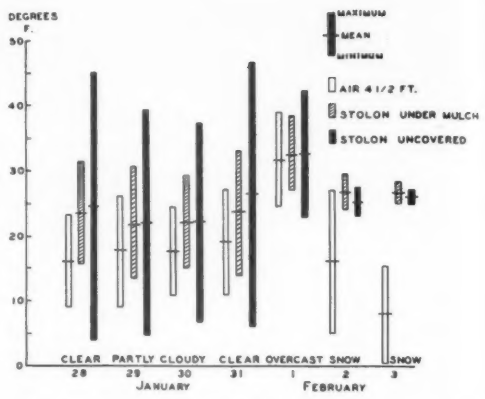

FIG. 4. Daily maximum, minimum and mean temperatures of air and clover stolons in the field under conditions of no cover, a grass mulch, and fresh snow. New Brunswick, New Jersey. 1955.

On some clear days uncovered stolons remained above freezing for 4 to 5 hours and reached maxima of 45 to 55° F. On the same days the maximum air temperatures at 137 cm above the ground were from 22 to 25° F, and the temperatures of stolons beneath a 5-cm grass mulch seldom exceeded 30° F. On clear winter nights the temperatures of uncovered stolons cooled more rapidly than, and reached minimum temperatures from 10 to 15° F lower than, stolons beneath a mulch (fig 4).

The surface of a grass or a snow cover absorbs radiant energy during the day and also insulates the plants beneath to avoid high maximum temperatures. Similarly these surfaces are cooled by outgoing radiation at night but the insulating properties of a grass or snow cover decreases cooling of the living plant material beneath it. Plant temperatures beneath as little as 4 cm of snow were more stable than any others observed (fig 4).

In plots where these temperature observations

were made, ladino clover over-wintering in a 10-cm stubble of orchardgrass made good growth in spring, whereas nearly all of the stolons not covered with a mulch were dead.

Summary

In laboratory studies cold-hardened ladino clover stolons and alfalfa roots and crowns were injured less severely when cooled slowly (2° F/hr) from 36 to 10 and 5° F than when cooled rapidly (7 to 10° F/hr) to the same temperature. Undercooling of 4 to 6° F was observed with slow cooling and a freezing point of 27° F was observed. Undercooling was not detected under conditions of a rapid drop in temperature and the freezing point was indistinct.

Variations in mid-winter plant temperatures in the field on clear days and nights were in excess of those employed in the laboratory and the rate of cooling approached that found lethal. On cloudy days and nights stolon temperatures were more stable. Highest daily maximum and lowest daily minimum plant temperatures existed where no grass or snow cover was present to shield the clover stolons. Daily maximum temperatures of clover stolons frequently exceeded 45° F while air temperatures 137 cm above the soil remained from 22 to 25° F. Growth in spring indicated that ladino clover stolons overwintering in a grass stubble survived well but nearly all were killed where no cover was present.

Acknowledgment is due Dr. E. R. Biel and Prof. A. V. Havens of the Department of Meteorology for cooperation in microclimate observations.

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IRON, THE LIMITING ELEMENT IN A CHLOROSIS: PART I. AVAILABILITY AND UTILIZATION OF IRON DEPENDENT UPON NUTRITION AND PLANT SPECIES[1,2]

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Iron is the limiting element in a chlorosis which develops in plants grown on some naturally calcareous soils. This chlorosis is caused by the failure of the susceptible plants to absorb iron from the soil or, in some cases, to utilize the iron which is absorbed (1, 2, 3, 8, 9, 10). The causative factors which act in the soil and plant to inactivate iron are not clearly understood, although several concepts relative to this problem have appeared in the literature (7, 11, 4, 14). Hewitt (8) reported a chlorosis in plants, typical of iron deficiency, which developed upon supplying a given level of cobalt, copper, zinc, nickel, chromium, manganese, or lead ions to sand cultures. Willis and Piland (16) found that copper affected the absorption and utilization of iron in corn and cotton, and Reuther et al (12) observed the same thing in citrus.

In this investigation, copper affected the absorption and utilization of iron in corn, but it had no affect in other plant species. When grown on calcareous soil plant species also differed in their susceptibility to an iron-deficiency chlorosis. Results reported in this paper show that iron metabolism differs between plant species and varieties and that these differences are dependent upon the growth media of the plant.

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[2] This work was supported in part by funds contributed by the Atomic Energy Commission.

Methods, Materials and Results

Absorption of Iron by Several Plant Species Differing in Their Susceptibility to Chlorosis: In a previous study (5) plants were grouped according to their susceptibility to iron or copper deficiency as follows: 1) Those plants which developed an iron-deficiency chlorosis when grown on a naturally calcareous soil, but did not develop copper-deficiency symptoms when grown on an organic soil low in available copper; 2) plants which developed copper-deficiency symptoms when grown on the organic soil but did not develop iron-deficiency chlorosis on the calcareous soil; 3) plants which showed retarded growth, slight deficiency symptoms in some cases, when grown on either the calcareous or organic soils; and 4) plants which showed no chlorosis or deficiency symptoms when grown on either soil. A plant was selected to represent each of these groups respectively: 1) PI-54619-5-1 soybeans, *Glycine Max*, 2) Thatcher spring wheat, *Triticum sativum*, 3) Minnesota Hybrid No. 800 corn, *Zea Mays*, and 4) Hawkeye soybeans, *Glycine Max*). These four plants were used to study the absorption and translocation of iron (Fe^{55-59}) as affected by two soils and two mixtures of these soils: 1) 3.2 kg calcareous soil, pH 7.9, 2) 1.8 kg calcareous soil and 0.8 kg organic soil, pH 7.5, 3) 0.8 kg calcareous and 1.0 kg organic soil, pH 7.2, 4) 1.8 kg organic soil, pH 6.0, 5) 1.8 kg organic soil plus 0.37 gm $CuSO_4 \cdot 5\,H_2O$. The calcareous soil

was a Millville silty clay loam. The copper deficient organic soil contained 86 % organic matter. The pH was determined in each of the soils and soil mixtures using a Beckman, Model H-2 pH meter. Soils 1 to 3 were mixed and fertilized with 1.2 gm of $NH_4H_2PO_4$ and 1.0 gm of KNO_3 per pot. Soils 4 and 5 received 1.3 gm of KNO_3, 1 gm of KH_2PO_4, 0.2 gm of KCl, 0.05 gm $ZnSO_4 \cdot 7\,H_2O$, 0.05 gm H_3BO_3 and 0.10 gm of $MnCl_2 \cdot 4\,H_2O$. Fifty microcuries of radioiron which had a specific activity of 4.55 mc/gm Fe was added to each pot of soil and soil mixture and equilibrated for three weeks. The soils were then seeded to their respective crops.

Autoradiographs were made (17) of the stalk and upper leaf of corn and the entire plant of soybeans and wheat. A meat slicer was used to cut the corn stalks into longitudinal sections about 0.32 cm thick. The third section from each of these cuttings, with the leaf, was mounted between botanical drying paper and placed between two 0.635 cm steel plates (22.86 cm × 20.32 cm). The specimens were dried in a force draft oven at 50° C in groups of five, with two lead bricks on each group. They were removed from the oven and the botanical drying paper was replaced with clean sheets of paper to avoid exposing the film to any expressed sap absorbed by the paper. The specimens were covered with a thin sheet of lumarith No. 15883 (cellulose acetate) to prevent the dried plant material from coming in direct contact with the X-ray film (Industrial Type-K). The film was placed upon the lumarith sheet in a dark room. Specimens and film were placed between two steel plates in a light tight box, so constructed as to allow stacking of the plates one upon the other, and left for five weeks. The film was then removed and developed.

Autoradiographs of the four plant species (fig 1) showed that wheat, corn and Hawkeye soybeans absorbed the radioiron from the calcareous soil. Leaf and flower primordia of the Hawkeye soybeans showed a greater accumulation of radioiron than the other plant parts. Less radioiron was absorbed by the chlorosis susceptible soybeans than any of the other plants. Much more radioiron was present in the roots of these soybeans than in the tops.

When two-thirds by volume of the soil mixture was organic soil (soil 3) there was an accumulation of iron in the nodes of corn (fig. 2) and these plants developed slight copper-deficiency symptoms. Less iron accumulated in the nodes of corn if copper was added to the growth media. Wheat developed severe copper-deficiency symptoms, but did not accumulate iron in the nodes. PI-54619-5-1 and Hawkeye soybeans, grown under the above conditions, did not develop copper-deficiency symptoms nor accumulate excessive iron in the plants.

Effect of Growth Media on the Translocation of Radioiron from the Cotyledons to Other Parts of Chlorosis Susceptible and Nonsusceptible Soybean Plants: Seeds containing radioiron were harvested from PI-54619-5-1 and Hawkeye soybean plants grown on sand-resin cultures that contained 50 μc of radioiron. No special significance was given to the sand-resin cultures in this investigation other than that they provided a growth medium with a good available supply of iron and other nutrients for plant growth. Separate quantities of cation resin (IR–100) were saturated respectively with K^+, Ca^{++} and Mg^{++} ions and anion resin (IR–4B) with NO_3^-, $H_2PO_4^-$ and $SO_4^=$. The resins were washed with demineralized water, dried and added to 5000 gm of sand so that 14 meq K^+, 42 meq Mg^{++}, 140 meq Ca^{++}, 96 meq NO_3^-, 32 meq $H_2PO_4^-$ and 64 meq $SO_4^=$ were added to each pot. Traces of microelements Mn, B, Cu, Mo, and Zn were added in solution when the sand cultures were watered.

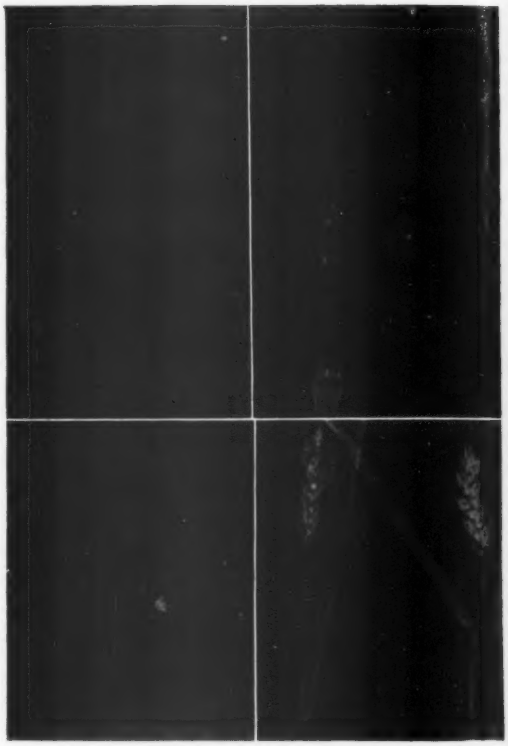

Fig. 1. Autoradiographs showing the ability of 4 plant species to absorb radioiron from a naturally calcareous soil: (*upper left*) Hawkeye soybeans, (*lower left*) PI-54619-5-1 soybeans were the only plants unable to absorb sufficient iron to prevent severe chlorosis, (*upper right*) Minn. Hybrid #800 corn, and (*lower right*) Thatcher spring wheat.

The seeds containing radioiron were planted in the soils and soil mixtures (previously described) to determine the soil effect on the distribution of iron within the plants. Four PI-54619-5-1 and four Hawkeye soybean plants were grown together in each pot. At 28 days, when the PI-54619-5-1 soybeans had developed chlorosis, the plants were harvested. Auto-

radiographs were made of the entire plant and the radioiron in various parts of the plant was determined to observe its distribution from the cotyledons.

Roots, stems, and leaves were harvested and prepared for radioiron analyses. No precautions were taken to remove all of the finer roots. They were brushed and washed free of soil with tap water and rinsed in demineralized water. Upper and lower leaves and stems were collected in separate groups. These plant materials were dried in a force draft oven at 70° C and ground in a Wiley mill to pass a 20-mesh sieve.

FIG. 2. Autoradiograph showing an accumulation of iron in the nodes of a corn plant which was deficient in copper.

TABLE I

EFFECT OF GROWTH MEDIUM ON THE UTILIZATION OF RADIOIRON IN THE COTYLEDONS AND MOVEMENT OF IRON INTO THE ROOTS OF 2 SOYBEAN VARIETIES

GROWTH MEDIUM	RADIOIRON CTS/SEC PER GM DRIED MATERIAL			
	COTYLEDONS		ROOTS	
	PI-54619-5-1	HAWKEYE	PI-54619-5-1	HAWKEYE
1. 3.2 kg calcareous soil	380	406	207	134
2. 1.8 kg calcareous + 0.8 kg organic soil	286	327	117	86
3. 0.8 kg calcareous + 1.0 kg organic soil	181	252	133	67

Radioiron was determined according to the method of Vasburgh, Flexner and Cowie (13). These data are not reported when autoradiographs are used to show the distribution of iron in the plant.

The seeds of these two soybean varieties contained approximately the same amount of radioiron. Table I shows comparative differences in the translocation of this iron from the cotyledons and the concentration of radioiron in the roots of these plants. Cotyledons from both soybean varieties retained approximately the same amount of radioiron, when grown on the calcareous soil. In the soil mixtures, more iron moved out of the cotyledons of the PI-54619-5-1 than the Hawkeye soybeans. Radioiron was more concentrated in the roots of PI-54619-5-1 than in the roots of Hawkeye soybeans. The cotyledons had dropped from the plants grown on the organic soil with and without copper and data are not reported for them.

The autoradiograph (fig 3) shows a greater movement of radioiron from the cotyledons to the upper leaf and flower primordia of the Hawkeye (nonchlorotic) than to the PI-54619-5-1 (chlorotic) soybeans. The Hawkeye soybeans were able to absorb soil iron; thus, both radioiron and soil iron moved into the upper leaves and flower primordia of these plants. The chlorosis susceptible soybeans were unable to absorb sufficient iron from the soil to prevent chlorosis, and the radioiron failed to be translocated, under these conditions, to the same extent as it was in the nonsusceptible soybeans.

INACTIVATION OF IRON WITHIN CHLOROSIS SUSCEPTIBLE PLANTS: In the previous experiments, insufficient iron was absorbed from the calcareous soil by the susceptible soybeans to prevent chlorosis. Thus, an approach graft technique was used to alleviate the chlorosis by supplying an available source of radioiron through the stems of the plants.

One group of chlorosis susceptible soybeans was grown on a naturally calcareous soil (no radioiron in

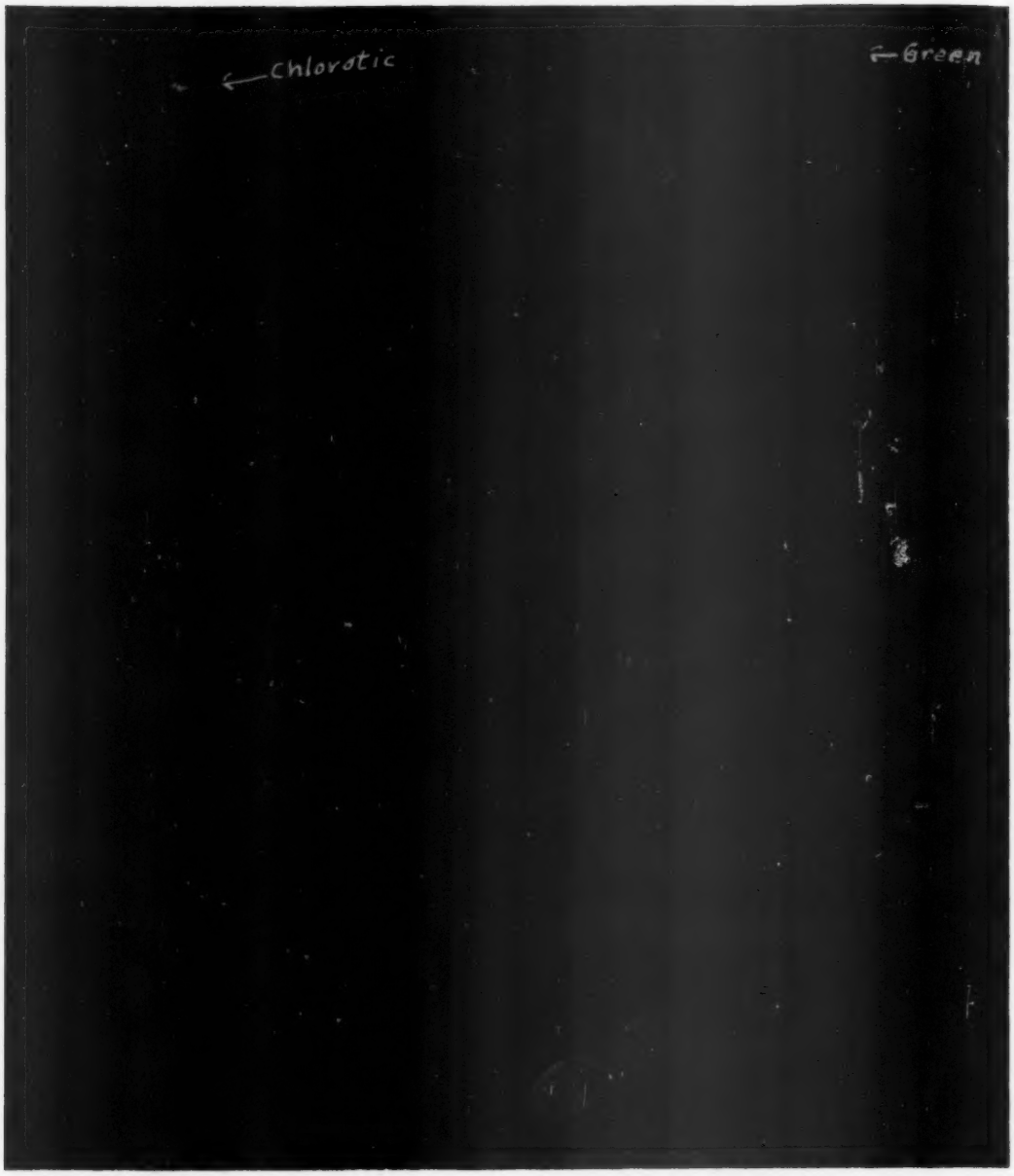

FIG. 3. Autoradiograph showing the distribution of radioiron from the cotyledons (only source of radioiron) in (*left*) PI-54619-5-1 and (*right*) Hawkeye soybeans grown on the calcareous Millville soil.

soil) and another group on a sand-resin culture (previously described) containing 50 μc of radioiron. When the first trifoliate leaves were about half developed and slightly chlorotic, soybeans growing on the calcareous soil were grafted to green soybeans growing on the sand-resin cultures. Within three weeks after the grafts were made three new green leaves had fully developed. The old chlorotic leaves remained chlorotic.

The tops of the soybeans growing on the sand-resin cultures were cut away from their roots leaving the tops of the two soybean plants dependent for survival upon the roots of the plants in the calcareous soil. Five days later the sixth trifoliate leaf was

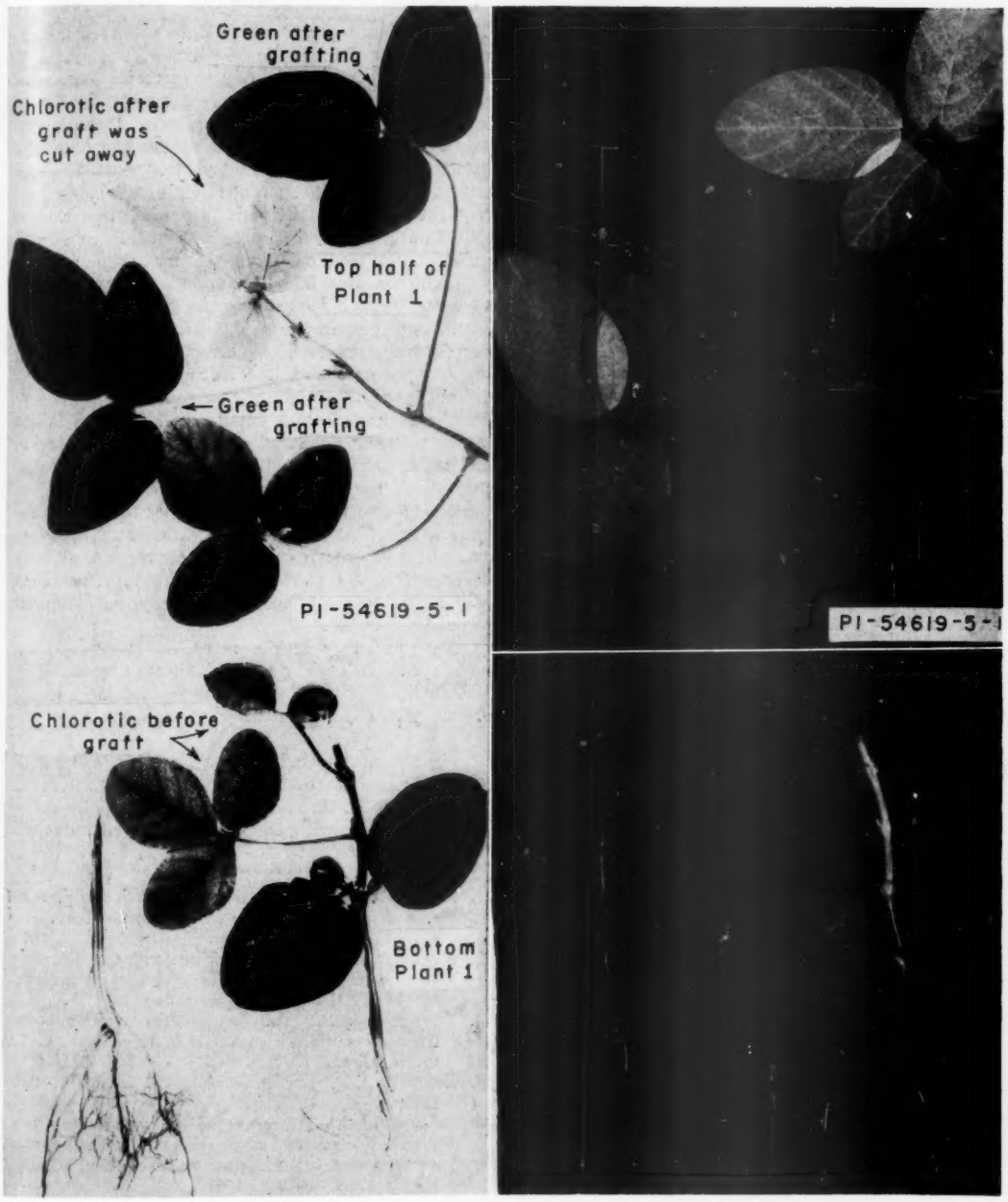

FIG. 4. Photographs (*on left*) and autoradiographs (*on right*) showing degree of chlorosis and distribution of radioiron in chlorotic and nonchlorotic leaves of PI-54619-5-1 soybeans. (*Lower left*) photograph of the lower half of the plant before iron became available through an approach graft. (*Lower right*) autoradiograph showing the distribution of iron in lower portion of the plant. (*Upper left*) photograph showing third, fourth, and fifth trifoliate leaves green, having developed after the graft became effective. The sixth trifoliate developed chlorosis when the available source of iron was stopped (graft cut away from root system). (*Upper right*) autoradiograph showing distribution of radioiron in the green and chlorotic leaves of the upper half of the plant.

chlorotic and about half developed. The plants were harvested and autoradiographs were made of them.

Autoradiographs (fig 4) show that radioiron was absorbed from the sand-resin culture and translocated through the approach graft into the plants growing on the calcareous soil. It was evident that the grafts were effective when the third trifoliate leaf became green. The first and second trifoliate leaves developed chlorosis before the grafts became effective and remained chlorotic and attached to the plant. In figure 4, at the left, are photographs of the lower and upper half of a plant which shows the extent of the chlorosis which developed. At the right are autoradiographs which show the distribution of radioiron in this plant. Radioiron moved into all parts of the plant, but more radioiron was located in the green leaves and stems than in the mesophyll of the chlorotic leaves. Radioiron also moved down into the roots. As soon as the external supply of iron was stopped (graft cut away), there was insufficient iron available from the calcareous soil or in the plant to prevent the new leaf which developed from becoming chlorotic. There was much less radioiron in this leaf than in the adjoining green leaves.

Discussion

A continuous source of available iron was necessary for the growth of all the plants in this investigation. But the concentration of available iron required in the growth media to prevent chlorosis differed between plant species and varieties. Each of the four plant species investigated differed in its growth response and ability to utilize iron from different growth media. The chlorosis susceptible PI-54619-5-1 soybeans were the only plants not able to absorb sufficient iron from the naturally calcareous soil to prevent chlorosis. Weiss (15) showed that the difference in efficiency of iron utilization between the chlorosis susceptible and nonsusceptible soybeans was conditioned by a recessive gene.

Neither of the soybean varieties showed any response to copper. Wheat developed severe copper-deficiency symptoms. Copper, not iron, appeared to be the more limiting element to the growth of wheat.

Copper affected iron metabolism in corn in a different manner than in either the soybeans or wheat. These results are in agreement with data obtained in previous investigations (5, 6) using catalase as an index of active iron in the plant. Catalase activity was comparatively lower in chlorotic than nonchlorotic plants and was markedly higher in copper-deficient than non-copper-deficient corn, tomatoes, and tobacco.

Inability of the plant to absorb iron from the soil, inactivation of iron in the plant, and the effect of copper on the utilization of iron are factors which appear to be coexistent with fundamental physiological and biochemical differences existing between plant species and varieties. An understanding of these differences in plant metabolism will probably lead to an understanding of the causative factors in chlorosis.

Summary

Four plant species which differed in their susceptibility to "lime-induced" chlorosis (iron deficiency) and copper deficiency were grown in soils of low available iron supply (calcareous) and soils of low available copper supply (organic). A continuous source of available iron appeared necessary for the growth of all the plants studied, but the plant species differed in the concentration of available iron required in the growth media to prevent iron chlorosis. One of the four species was unable to absorb sufficient iron from calcareous soil for normal growth.

Radioiron was supplied to chlorosis susceptible soybeans through approach grafts and seed. In each case, insufficient iron was absorbed from the soil to prevent chlorosis when the available source of iron was stopped or depleted. The radioiron in the plant did not move appreciably under these conditions.

The available copper supply in the growth media seemed to have a marked effect upon the absorption and utilization of iron by corn. Iron accumulated throughout the copper-deficient plant (especially in the nodes) and the addition of copper to the soil decreased this absorption. Both varieties of soybeans and wheat differed from corn in this respect. Wheat developed severe copper-deficiency symptoms, corn only moderate symptoms, and the soybeans did not respond to copper.

The physiology of the plant, as determined by plant species or variety and as influenced by the mineral nutrition of the soil, determines in a large measure the status of iron in the plant.

The authors are indebted to Harry T. Borthwick for his helpful instructions in making the approach grafts, and to D. W. Thorne and J. F. Davis for sending us the calcareous and organic soils, respectively.

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IRON, THE LIMITING ELEMENT IN A CHLOROSIS: PART II. COPPER-PHOSPHORUS INDUCED CHLOROSIS DEPENDENT UPON PLANT SPECIES AND VARIETIES[1]

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The purpose of this investigation was to study the effect of varying rates of copper and phosphorus upon the absorption and translocation of iron and the incidence of chlorosis in several plant species known (2, 3) to vary in their susceptibility to chlorosis, and to make comparative studies between chlorosis-susceptible and nonsusceptible soybean plants as to the iron concentration required in solution to prevent chlorosis.

METHODS, MATERIALS AND RESULTS

EFFECT OF INCREASING RATES OF COPPER AND PHOSPHORUS UPON THE INCIDENCE OF CHLOROSIS: Six levels of copper sulfate, 0, 25, 50, 100, 200, and 400 ppm; and 5 levels of P_2O_5 as phosphoric acid: 0, 100, 200, 400, and 800 ppm were applied to soil mixtures consisting of 0.80 kg calcareous soil and 1.0 kg organic soil. This soil mixture gave a growth medium relatively low in available Cu and P. Each pot received 1.0 gm of KNO_3. Thatcher spring wheat (*Triticum sativum*), Colora rice (*Oryza sativa*), PI-54619-5-1 and Hawkeye soybeans (*Glycine max*) were grown on each treatment. The wheat and rice were grown at different times, but the two soybean varieties were grown at the same time in the same pots. Eight soybean plants were grown in each pot, 4 plants each of PI-54619-5-1 and Hawkeye.

The wheat was harvested 35 days after planting when Cu-deficiency symptoms had fully developed on some of the treatments. The Cu deficiency was first evidenced by a retardation in growth and a light green color of the foliage. This was followed by a curling and dying back of the leaf tips, with subsequent loss of chlorophyll and drying of the leaves. Rice plants were harvested 50 days after planting when Fe chlorosis had developed in plants most deficient in Fe. Heads were not produced. Spectrochemical analyses were made for the ten elements (1).

The two soybean varieties were harvested when the yellow color of the leaves indicated that iron chlo-

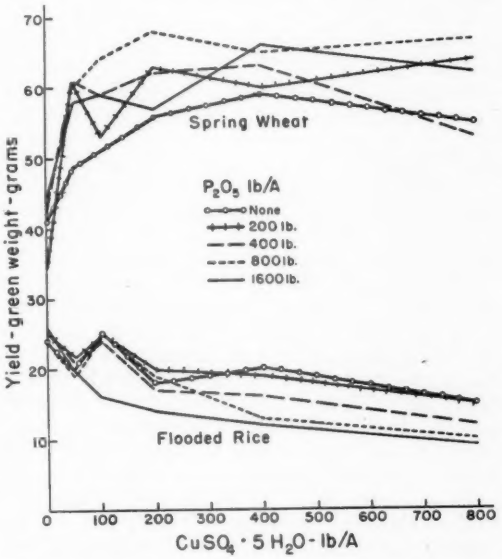

FIG. 1. The effect of increasing rates of P and Cu upon the yield of wheat and rice grown on a soil mixture.

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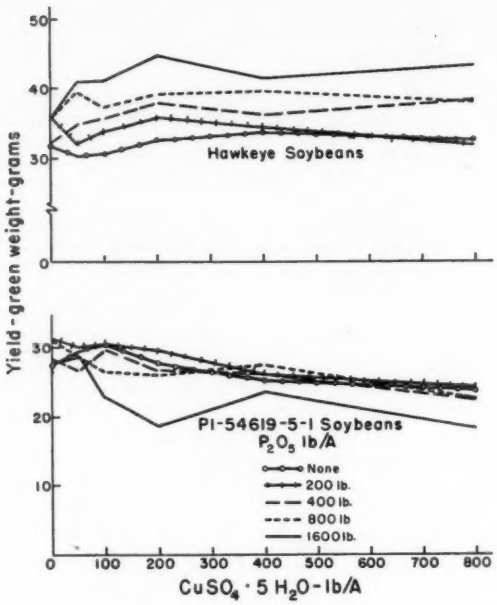

FIG. 2. The effect of increasing rates of P and Cu upon the yield of Hawkeye and PI-54619-5-1 soybeans grown on a soil mixture.

rosis had developed on some of the treatments (31 days). Four plants from each treatment were frozen at – 10° C overnight, thawed out the following morning, and the sap squeezed from the plants with a press. The pH of the plant sap was determined immediately after the sap was squeezed from the plant with a Carver press and the amount of sap extracted was recorded. The residue was placed in tared beakers, dried at 80° C, and the dried residue was weighed and recorded. Sap and residue were each treated with 10 ml of HNO_3 acid and digested to dryness. The residues were ashed in a muffle furnace at 500° C overnight and then digested in 3.0 ml of 8 *N* HCl on a warm hot plate for about 30 minutes. Iron and P were determined in an aliquot of each of these solutions, using the methods of Saywell and Cunningham (11) and Dickman and Bray (4), respectively.

Figures 1 and 2 show the yields obtained from the above plants as affected by varying rates of Cu and P. These yield curves show that the two chlorosis-susceptible plants, rice and PI-54619-5-1 soybeans, decreased in yield with increasing Cu and P applications. The yields from the chlorosis-susceptible plants were the least on the highest phosphate treatment, where, in contrast, the yields of wheat and Hawkeye soybeans (chlorosis-nonsusceptible plants) were the greatest. Increased Cu applications decreased the yield of the chlorosis-suceptible plants, but did not decrease the yields of the nonsusceptible plants. Copper and phosphorus were increasingly more effective in producing chlorosis if applied together than if either element was applied separately.

Wheat was the only plant that developed Cu-deficiency symptoms, resulting in decreased yields, when $CuSO_4$ was not added to the soil mixture.

Table I shows the effect of Cu and P applied to the soil mixture at different rates and ratios upon the concentration of Fe in the leaves of wheat and rice and the total Fe in the two soybean varieties. There was no marked decrease of Fe in any of the plants with increasing rates of $CuSO_4$ up to and including 200 ppm as long as phosphorus was *not* included in the treatment. At the 50, 100, and 200 ppm $CuSO_4$ treatments, the Fe in the chlorosis-susceptible plants (rice and PI-54619-5-1 soybeans) decreased on the high phosphate treatments as the rate of $CuSO_4$ applied increased. The phosphorus treatments had no effect upon the absorption of Fe in rice and PI-54619-5-1 soybeans if $CuSO_4$ was *not* added to the soil mixture. Applied together, these two elements decreased the amount of Fe absorbed and translocated to the

TABLE I

EFFECT OF P AND CU ON THE ABSORPTION OF IRON BY SEVERAL PLANT SPECIES THAT DIFFERED IN THEIR SUSCEPTIBILITY TO CHLOROSIS

TREATMENTS		Fe			
		WHEAT LEAVES	RICE LEAVES	SOYBEANS	
P_2O_5	$CuSO_4 \cdot 5\ H_2O$			PI *	H *
ppm	*ppm*	*ppm*	*ppm*	*μgm* **	
0		85	..	291	351
100		94	81	229	474
200	0	100	79	290	405
400		110	78	282	476
800		200	83	243	509
0		110	82	300	384
100		120	66	290	392
200	25	120	74	275	455
400		110	71	258	450
800		120	76	167	531
0		110	81	291	406
100		120	74	275	440
200	50	110	78	235	436
400		120	83	204	435
800		140	53	152	516
0		140	72	267	424
100		130	83	263	438
200	100	120	65	187	360
400		140	40	118	471
800		110	34	158	491
0		96	75	228	...
100		180	63	219	419
200	200	120	49	207	471
400		100	25	192	429
800		100	28	182	526
0		130	37	160	330
100		110	37	168	351
200	400	110	31	152	368
400		110	30	187	478
800		160	55	134	568

* PI = PI-54619-5-1 soybeans.
H = Hawkeye soybeans.

** Micrograms of iron absorbed by 4 plants (plant sap + plant residue), harvested when third trifoliate leaf was partially developed.

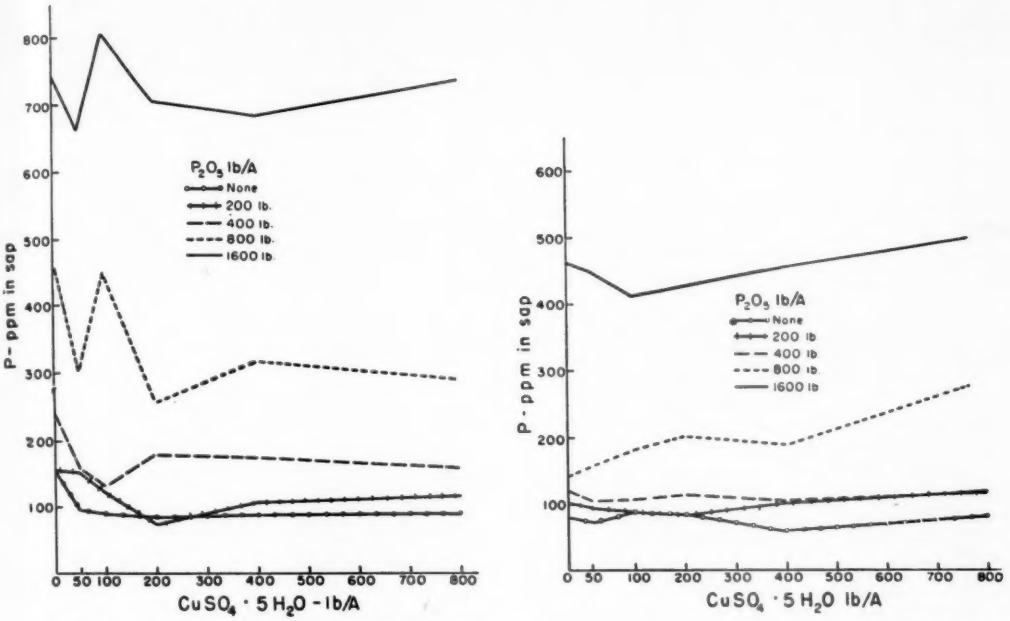

FIG. 3. The effect of increasing rates of P and Cu upon the conc (ppm) of P in the plant sap of (*left*) PI-54619-5-1 and (*right*) Hawkeye soybeans grown on a soil mixture.

above-ground portions of the chlorosis-susceptible plants. The highest Cu and P applications applied separately or together did *not* decrease the absorption of Fe by wheat and Hawkeye soybeans.

Sap from PI-54619-5-1 soybean plants grown on the highest phosphate treatment had a pH slightly below 6, while that from the Hawkeye variety was slightly above pH 6. The pH of the sap in both varieties was lower on the high phosphate than on the low phosphate treatments. The rates of Cu applied did not have much effect upon the pH of the sap extracted. The pH of the soil mixture did not vary between treatments.

At the 200, 400, and 800 ppm P_2O_5 treatments (fig 3), the concentration of P in the sap was greater in the susceptible than in the nonsusceptible soybeans. This difference was greatest where the highest rate of P was applied. Phosphorus concentration in the residue of these plants increased with treatment, but did not differ between soybean varieties. Increasing the amount of Cu applied to the soil mixture did not have much effect upon the concentration or total P in the sap and residue.

EFFECT OF CHELATE ON THE INCIDENCE OF CHLOROSIS AND THE DISTRIBUTION OF PHOSPHORUS AND IRON IN SOYBEAN PLANTS: Hawkeye and PI-54619-5-1 soybeans were grown on the calcareous Millville soil with 5 rates of P: 0, 100, 200, 400, and 800 ppm P_2O_5. Four pots were made up for each treatment. One-half of the pots in each treatment received 150 ppm of chelate DTPA (diethylenetriamine pentaacetic acid).

The plants were harvested, frozen overnight at −10° C, sap pressed from the plants, pH of sap determined, and the Fe and P determined in the sap and residue as described above.

The P concentration was lower in the sap of both varieties of soybeans when DTPA was added to the calcareous soil (fig 4). DTPA alleviated chlorosis and increased the yield of PI-54619-5-1 soybeans, but it did not affect the yield of the nonchlorotic Hawkeye soybeans. There appeared to be no relationship be-

TABLE II

CONCENTRATION AND TOTAL FE IN THE SAP AND RESIDUE OF 2 SOYBEAN VARIETIES GROWN ON A CALCAREOUS SOIL WITH AND WITHOUT CHELATE (DTPA)

Treatments P_2O_5 ppm	Chelate				No chelate			
	Sap		Residue		Sap		Residue	
	ppm	µgm	ppm	µgm	ppm	µgm	ppm	µgm
PI-54619-5-1 soybeans								
0	4.2	50	71	133	3.4	24	62	93
100	3.1	76	91	265	2.9	39	61	75
200	3.5	45	93	210	2.4	32	57	90
400	3.3	65	71	165	3.2	25	62	83
800	3.3	69	66	178	5.4	17	61	83
Hawkeye soybeans								
0	4.2	83	97	285	5.2	40	79	225
100	4.4	65	89	230	5.2	69	65	160
200	4.1	69	85	290	3.7	53	70	210
400	3.7	76	98	315	4.8	42	59	165
800	3.7	69	93	310	3.5	50	65	195

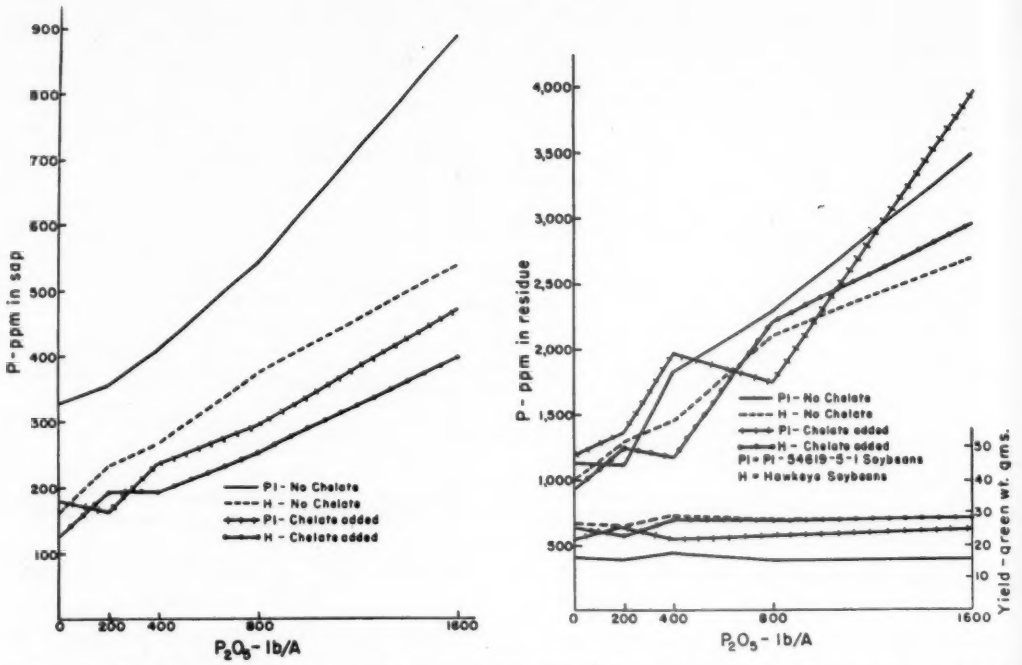

FIG. 4. The effect of increasing rates of P upon the conc of P in the sap and residue of PI-54619-5-1 and Hawkeye soybeans grown on a calcareous soil with and without added chelate (DTPA).

tween the amount of P which was applied to the soil and the concentration of Fe in the sap and residue (table II).

IRON CONCENTRATION REQUIRED IN SOLUTION CULTURES FOR THE GROWTH OF CHLOROSIS-SUSCEPTIBLE AND NONSUSCEPTIBLE PLANTS: A comparative study was made of the Fe concentration required in solution cultures (13, 14) for the growth of wheat, corn, PI-54619-5-1 and Hawkeye soybean plants. Two sources of Fe were used: 1) DTPA-Fe and 2) $FeCl_3 \cdot 6\,H_2O$. Seven levels of Fe were used in each case: 0, 0.1, 0.2, 0.4, 1.0, 4.0, and 8.0 ppm. The plants were harvested after Fe became a limiting factor to growth and chlorosis had fully developed. The 2 soybean varieties were grown in the same pots; 2 plants each of PI-54619-5-1 and Hawkeye soybeans.

The growth response of wheat, corn, PI-54619-5-1 and Hawkeye soybeans differed with a limited supply of available Fe. All of the plants showed some chlorosis on the 0.4 ppm of $FeCl_3 \cdot 6\,H_2O$ treatment. Terminal growth of PI-54619-5-1 soybeans and corn became first chlorotic, then necrotic, and finally the entire plant gradually dried up. The terminal growth in wheat remained green but the older leaves became chlorotic, necrotic, and then dry. Hawkeye soybeans were partially chlorotic, but at the time of harvest the terminal portions of the plants were still growing.

Table III shows the yield response of the two soybean varieties to both inorganic and chelated iron applied at different rates. A greater concentration of both inorganic and chelated iron was required in the solution cultures by PI-54619-5-1 than by Hawkeye soybeans to give the same growth response. Inorganic iron was almost as effective as chelated iron in the growth of Hawkeye soybeans. The pH of the solution cultures at the beginning of the experiment were approximately 5.5 and increased, where the plants made good growth, to approximately pH 7.0 during the course of the experiment.

FIELD STUDIES

Several western states were visited during the summer of 1953 to study the causative factors of chlorosis associated with some of the calcareous soils of that area. Peach growers generally had observed that if a green manure crop was disced into the soil and the soil irrigated, peach trees developed chlorosis (yellows). Two such orchards were located in an irrigated valley in the West. There was a small area in each orchard which did *not* have a green manure crop and the trees were green in these areas. Leaf samples were taken from chlorotic and nonchlorotic peach trees located about 50 yards apart in these orchards. The leaves were rinsed in distilled water, each leaf individually washed with 0.1 *N* HCl and then washed again with distilled water. A forced draft oven was used to dry the leaves which were then ground in a Wiley mill to pass a 40-mesh sieve. Spectrochemical analyses were made for ten elements: Cu, Mn, Fe, Al, B, P, Na, Mg, Ca, and K. The pro-

TABLE III

YIELD OF HAWKEYE AND PI-54619-5-1 SOYBEANS GROWN IN SOLUTION CULTURES CONTAINING 2 TYPES OF FE AT DIFFERENT CONCENTRATIONS

TREATMENTS FE	YIELD—DRY WT/2 PLANTS			
	HAWKEYE		PI-54619-5-1	
	DTPA-FE	$FeCl_3 \cdot 6 H_2O$	DTPA-FE	$FeCl_3 \cdot 6 H_2O$
ppm	*gm*	*gm*	*gm*	*gm*
0.0	0.68	None	0.50	None
0.1	0.72	0.56	0.69	0.63
0.2	3.51	1.44	1.94	1.11
0.4	6.25	7.17	4.24	0.96
1.0	7.48	6.49	8.32	1.14
4.0	8.19	7.44	14.94	2.16
8.0	11.24	12.38	11.88	8.03

cedure used was that developed by the Plant Industry Station, Spectrochemical Laboratory (1). The results are an average of 5 samples.

Chlorotic leaves had a higher concentration of Cu, B, and P; and less Fe, Al, and Ca than nonchlorotic leaves (table IV).

To check the observations which were made in the field concerning the effects of a green manure crop on the development of chlorosis, Telephone (dark podded) peas were grown in the soil mixture described above. The plants were harvested when they were about 20 cm high and cut into small pieces. These pieces were mixed thoroughly into the soil and then 4 PI-54619-5-1 and 4 Hawkeye soybeans were planted in each pot. The plants were harvested after chlorosis had developed (30 days). All of the leaves were removed from the plants and dried in a forced draft oven. The ten elements were determined spectrochemically in the leaves as previously described.

Chlorosis-susceptible PI-54619-5-1 soybeans developed chlorosis where the green manure crop was mixed into the soil mixture. The chlorotic leaves had a higher concentration of Cu and P and less Fe than nonchlorotic leaves (table V). Comparative data are also given in this table for the Hawkeye soybeans which did not develop chlorosis nor show any change in Cu and P concentrations in the leaves. Chlorosis did not develop in either variety of soybean where a green manure crop was not used and the mineral composition of their leaves did not vary appreciably between varieties.

DISCUSSION

Microelement imbalance has been observed (5, 7, 8, 10, 12, 16) to be a causative factor in Fe chlorosis. Phosphorus has been reported (6, 9) to exert a marked influence on the absorption and utilization of Fe. Under the conditions of the experiment reported here, P applications to the growth medium induced iron chlorosis *only* if Cu was also applied. That is, P and Cu were much more effective in causing Fe chlorosis if applied together than if either element was applied alone. The rate of Cu application, however, did not affect the P accumulation in either the sap or residue.

In respect to the incidence of chlorosis, there was no difference in the P concentration of the residues from either chlorotic or nonchlorotic soybeans. A P differential, however, was found in the sap portion, there being more of the element in sap from chlorotic than in that from the nonchlorotic plants. The Fe concentration in the sap was not related to the P in the sap in these experiments. But other experiments with chlorotic lupines and with Cu-deficient corn (unpublished data) showed a relationship between the accumulation of P and of Fe in the respective plants.

A Cu–P effect upon the utilization of Fe from the growth media by chlorosis-susceptible soybeans was apparent, in contrast, to no effect upon the chlorosis-nonsusceptible soybeans. This difference was very marked and is known to be genetically controlled (15). It may explain the reasons why chlorosis-susceptible soybeans require a greater concentration of available Fe in the growth media to prevent iron chlorosis than the nonsusceptible variety.

Iron chlorosis appears to be caused by several different factors that are dependent upon the physiology of the plant species. In comparing Thatcher spring wheat with PI-54619-5-1 soybeans, the difference in susceptibility to chlorosis might be due to the effect of the dominance of specific metabolic systems, the one requiring Cu in wheat and the other requiring Fe in soybeans. Copper and P together decreased the absorption and utilization of Fe in the PI-54619-5-1 soybeans, but did not have this effect in wheat or in

TABLE IV

SPECTROCHEMICAL DATA SHOWING THE EFFECT OF GREEN AND BARNYARD MANURE ON THE MINERAL COMPOSITION OF PEACH LEAVES

TREATMENTS *	ELEMENTS IN CHLOROTIC AND NONCHLOROTIC PEACH LEAVES—PPM									
	CU	MN	FE	AL	B	NA	P	MG	CA	K
Orchard A **—GM	17.4	53	91	123	112	48	4200	8500	16000	39000
Orchard A—No GM	11.4	40	230	324	79	44	1800	11000	28000	31000
Orchard B **—WBY	14.0	35	73	78	100	41	3100	7200	14000	32000
Orchard B—No WBY ...	11.0	53	138	151	91	46	2700	7700	26000	32000

* GM—Green manure crop of peas disced into soil. WBY—Weeds and barnyard manure disced into soil.
** Chlorotic leaves.

TABLE V

EFFECT OF A GREEN MANURE CROP ON THE MINERAL COMPOSITION OF PI-54619-5-1 AND HAWKEYE SOYBEAN LEAVES GROWN ON A SOIL MIXTURE OF 0.80 KG CALCAREOUS SOIL AND 1.0 KG ORGANIC SOIL, pH 7.3

TREATMENTS	PLANT	ELEMENTS IN LEAVES—PPM										YIELD ** GM
		Cu	Mn	Fe	Al	B	Na	P	Mg	Ca	K	
No green manure crop	PI *	3.0	28	85	45	36	22	2000	5800	11000	29000	24
	H *	3.6	21	110	48	38	19	1800	5400	12000	29000	26
Peas as green crop	PI	5.3	17	45	20	45	19	3500	5300	11000	33000	15 †
	H	3.2	16	80	18	35	18	1700	5400	13000	26000	22

* PI = Chlorosis-susceptible PI-54619-5-1 soybeans. H = Chlorosis-nonsusceptible Hawkeye soybeans.
** Yield/4 plants—green wt.
† Chlorosis developed in these plants.

Hawkeye soybeans. The latter plants showed no response to Cu and did not develop Cu-induced iron chlorosis. Thus, each of the above plants appears to differ physiologically from the other. These results emphasize the complexities in the chlorosis problems in which Fe metabolism is involved. It seems possible, too, that the responses noted here are applicable to other physiological problems in plant nutrition.

SUMMARY

Field and greenhouse experiments showed that the elements most consistently affected in a comparison of chlorotic and nonchlorotic leaves were Cu, P, and Fe. Cu and P were higher and Fe lower in chlorotic than in nonchlorotic leaves.

Under the conditions of these experiments, increasing additions of P did *not* affect the absorption or utilization of Fe, to the extent that chlorosis developed, unless Cu was also present in the growth media. Copper and P were more effective in producing chlorosis if applied together, than if either element was applied separately. Plant species and varieties investigated differed in their susceptibility to this Cu–P induced Fe chlorosis.

The concentration of P was higher in the sap of chlorotic than nonchlorotic soybeans. It did not differ appreciably in the residue of these plants. More P was absorbed by the plants as the rate applied to the growth medium increased. This resulted in a proportionately greater concentration of P in the sap than in the plant residue.

Chlorosis-susceptible and nonsusceptible plants differed in their Fe requirements and deficiency symptoms when grown on solution cultures with different kinds and rates of Fe as variables. Chlorosis-susceptible soybeans required a higher concentration of both inorganic and chelated iron in the solution cultures than the nonsusceptible variety to prevent Fe-deficiency chlorosis.

Several factors are discussed with reference to the cause and susceptibility of plant species to chlorosis.

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RESPIRATION OF THE PEA PLANT. METABOLISM OF HEXOSE PHOSPHATE AND TRIOSE PHOSPHATE BY CELL-FREE EXTRACTS OF PEA ROOTS[1,2]

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The urge to characterize the carbon transformations in photosynthesis has rapidly resulted in much evidence for the presence of two pathways of carbohydrate metabolism, the Embden-Meyerhof-Parnas and the direct oxidation pathway in the green leaves and seeds of higher plants (2, 3, 4, 6, 8, 11, 13, 24). However, investigations concerning these pathways in other portions of the plant have been limited (6, 7, 12, 25).

The present investigation was undertaken to establish whether the primary enzymes of the two pathways are present in the roots and, if so, whether the pathways are similar to those in chlorophyll-containing tissue. The pea plant (12 to 15 days) was selected for this study.

EXPERIMENTAL MATERIALS

Glucose-6-phosphate (G-6-P) was prepared as the heptahydrate of the barium salt by the procedure of Seegmiller and Horecker (21). The crude salt was dissolved in a small amount of HCl, adjusted to pH 8.0 with saturated $Ba(OH)_2$ and left overnight at 4 to 5° C. The insoluble heptahydrate which crystallized out was washed with ethanol and acetone. Enzymatic analysis (17) showed it to be 99.3 % pure.

Fructose-6-phosphate (F-6-P) was purchased from the Schwarz Laboratories as the barium salt. It was found free of G-6-P and FDP.

A commercial preparation of the barium salt of fructose-1,6-diphosphate (FDP) was converted to the potassium salt and the contaminating inorganic phosphate removed with magnesia mixture. The product used was free of inorganic phosphate and contained 97.5 % FDP as determined enzymatically (20). This FDP was used in the aldolase experiments. In all other experiments involving FDP, the commercial preparation was used.

The barium salt of 6-phosphogluconate (6-PG) was prepared by the method of Seegmiller and Horecker (21). Enzymatic analysis indicated 82 % 6-PG and traces of G-6-P.

Phosphoglyceric acid (PGA) was purchased from the Schwarz Laboratories. Phosphorus analysis (total phosphorus-hydrolyzable phosphorus) showed it to be at least 99 % pure.

Triphosphopyridine nucleotide (TPN) was prepared by the method of LePage and Mueller (18). It contained 31 % TPN (analyzed with G-6-P dehydrogenase) and no DPN (analyzed with alcohol dehydrogenase). A sample (72 %) purchased from the Sigma Chemical Company was also used.

Diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) were purchased from the Schwarz Laboratories.

Lactic acid dehydrogenase, G-6-P dehydrogenase, phosphohexose isomerase and the α-glycerol phosphate dehydrogenase mixture (used to assay FDP) were kindly supplied by Dr. B. L. Horecker. Aldolase was kindly supplied by Dr. E. Racker.

METHODS

PREPARATION OF PEA ROOT EXTRACTS: *Pisum sativum* variety Alaska (Roger Brothers Seed Co., Idaho Falls, Idaho) was used throughout this investigation The seeds were soaked overnight in tap water at room temperature, then transferred to flats containing acid-washed sand or gravel. The material was watered daily with tap water. The temperature in the house was 16 to 20° C. During the winter (November through March) the solar radiation was supplemented with artificial light from 8 A.M. to 5 P.M. However, during periods of little solar radiation, some batches of plants became etiolated; these plants were not used. The plants were similar to those used in a previous investigation (11). The roots were cut off slightly below the cotyledons. They were washed in tap water, followed by a washing in distilled water. Extracts were made of this material in two ways: 1) 70 gm of roots were ground in the cold room (4° C) with an equal weight of acid-washed, cold, sharp sand and 5 ml of cold 0.5 M $KHCO_3$ and 5 ml ethylene diamine tetra-acetic acid (versene), pH 8.0 (30 mg/ml). The brei was strained through 4 thicknesses of cheesecloth and centrifuged at 18,000 × g for 20 minutes. The supernatant solution had a light tan color, its volume was 50 to 100 ml and its pH was approximately 7.6. This preparation will be designated hereafter as extract I. 2) Approximately 50 gm of roots were blended for 1 minute in a chilled Waring blendor with 400 ml of acetone cooled to −18° C. The homogenate was filtered rapidly with suction. The dry powder was reblended with another 300 ml of acetone. When dry, the powder was screened through a plastic No. 20 sieve, then stored at −18° C in a desiccator containing P_2O_5. Extracts were made by grinding the powder with 0.1 M tris (hydroxymethyl)-amino-methane (TRIS) buffer pH 8.0 for 30 minutes at room temperature. The mixture was centrifuged at 18,000 × g for 20 minutes. On occasion the supernatant material was passed through glass wool. This preparation is extract II.

ANALYTICAL DETERMINATIONS: Inorganic phosphate was determined by the procedure of Fiske and Subbarow (10). Inorganic phosphate liberated by

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hydrolysis during 20 minutes in 1 *N* KOH at room temperature was used as a measure of triose phosphorus. Iodine oxidation to differentiate the trioses was carried out according to Stumpf (23). The method of Mejbaum (19), modified by extending the heating period to 40 minutes, was used to determine pentose; D-arabinose (Pfanstiehl) was used as standard. G-6-P was determined by its ability to react with TPN in the presence of G-6-P dehydrogenase. F-6-P was determined in a similar manner with the addition of phosphohexose isomerase. FDP was determined by a method of Racker (20).

ESTIMATION OF DEHYDROGENASE ACTIVITY: Dehydrogenase activity was determined by following the rate of reduction of TPN or DPN at 340 mμ in 1-cm cells in a Beckman spectrophotometer, model DU, at room temperature. Usually the cell contained 0.2 ml extract, 10 μM of $MgCl_2$, 0.1 μM of TPN or DPN, 125 μM of glycylglycine buffer or 100 μM of TRIS buffer in a total volume of 3.0 ml. The reaction was started by the addition of 0.1 ml substrate to both cells, the blank cell lacking TPN or DPN. The inhibitor studies were carried out by incubating the test system with the poison for 5 minutes before addition of substrate.

RESULTS AND DISCUSSION

ENZYMES OF THE DIRECT OXIDATIVE PATHWAY, *G-6-P and 6-PG Dehydrogenases:* As demonstrated for most material (2, 11, 14), the direct oxidative pathway consists of two TPN-linked dehydrogenases which converts G-6-P to pentose phosphate and CO_2 via 6-PG. Spectrophotometric data indicate that the cell-free preparations of pea root also possess TPN-specific enzymes catalyzing this pathway.

Properties of G-6-P and 6-PG Dehydrogenases: The pH-activity curves for both dehydrogenases, using extracts of acetone powders, were determined in 0.04 M glycylglycine. While G-6-P dehydrogenase has an optimum pH at approximately 7.8, 6-PG dehydrogenase has its highest activity at approximately pH 7.5. The pH optimum for G-6-P dehydrogenase is similar to that found for yeast (16) and rat liver (14). The pH optimum for 6-PG dehydrogenase is close to the value of 7.6 found for purified yeast 6-PG dehydrogenase by Horecker and Smyrniotis (16), but is in contrast to the high pH optimum of 9.0 for the rat liver enzyme (14). To rule out TPN reduction by further products of 6-PG metabolism, ribose-5-phosphate (3.4 μM) was tested under the same conditions; no TPN reduction occurred.

The Effect of Inhibitors and Activators on the Dehydrogenases: Mg^{++} (3×10^{-2} M) doubles the rate of G-6-P dehydrogenase while 6-PG dehydrogenase activity is increased by one-half. High concentrations of iodoacetamide (1×10^{-2} M) inhibit both dehydrogenases slightly (20 %). However, the dehydrogenases are inhibited completely by 1×10^{-3} M *p*-chloromercuribenzoate. These data are similar to those reported for the liver dehydrogenases (14).

Stoichiometry of 6-PG Oxidation: The crude enzyme preparations used in these studies possess enzymes capable of reoxidizing DPNH but reoxidize TPNH slowly or to a limited extent. In order to accumulate the reaction products and avoid the use of stoichiometric amounts of TPN it was necessary to furnish a mechanism for the oxidation of the reduced coenzyme. This was accomplished by coupling the oxidation reaction to the reduction of pyruvate to lactate in the presence of rabbit muscle lactic acid dehydrogenase. The equations for the coupled systems are:

$$\text{6-Phosphogluconate} + \text{TPN} \longrightarrow \text{pentose phosphate} + CO_2 + \text{TPNH}$$

$$\text{Pyruvate} + \text{TPNH} \longrightarrow \text{lactate} + \text{TPN}$$

In agreement with these equations the data in table I show that for each mole of CO_2 and pentose phosphate formed, a mole of pyruvate is reduced. The reduction of pyruvate is accomplished by a stoichiometric amount of lactate, determined by the procedure of Barker and Summerson (5). The appearance of small amounts of F-6-P (0.5 μM) and traces of G-6-P (0.03 μM) indicates a further metabolism of the pentose phosphate in a manner previously described (13). Similar data have been obtained with turnip root (6) by coupling the oxidation to the reduction of glutathione.

Recently a second pathway of 6-PG oxidation (9, 26) alternate to that catalyzed by 6-PG dehydrogenase has been described. Attempts to find this second pathway in our pea root preparations have been unsuccessful.

GLYCOLYTIC ENZYMES, *The Conversion of FDP to Hexosemonophosphate (HMP):* In TRIS extracts of acetone powder, FDP is utilized and HMP is formed (fig 1). The utilization of FDP is considerably more rapid than the formation of HMP. The addition of Mg^{++} resulted both in a more rapid utilization of FDP and in a greater formation of HMP. Magnesium probably activates a FDP phosphatase. Further analysis of the HMP showed it to contain 72 % G-6-P

TABLE I

STOICHIOMETRY OF PHOSPHOGLUCONATE OXIDATION

CONC	PHOSPHOGLUCONATE μM	PYRUVATE μM	CO_2 μM	PENTOSE μM	LACTATE μM
Initial	12.0	24.0	..	4.4	...
Final	2.0	15.0	8.2	11.7	11.1
Δ	10.0	9.0	8.2	7.3	11.1

The test system contained, in addition to the initial quantities shown in the table: 100 μM glycylglycine buffer, pH 7.5, 0.31 μM of TPN, 20 μM of $MgCl_2$, 0.3 mg of lactic acid dehydrogenase, and 1.0 ml extract I, in a total volume of 2.05 ml. The gas space contained nitrogen; temperature 30° C. The reaction was begun by tipping 6-PG and TPN from one side arm and stopped by addition of 0.2 ml of 50 % TCA from a second side arm. Total CO_2 includes CO_2 liberated by the TCA. The figures in the table are corrected for endogenous reactions by running a control in which phosphogluconate was omitted.

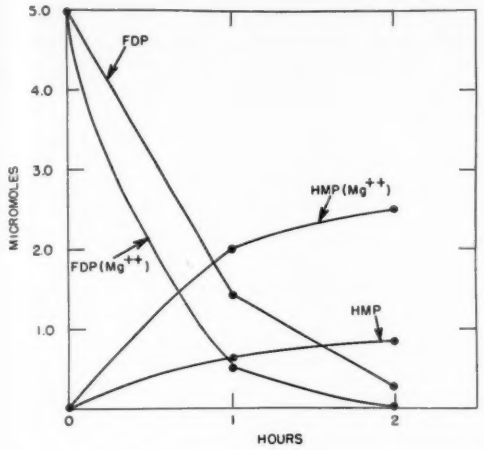

FIG. 1. Hexose monophosphate formation from fructose diphosphate. The incubation mixtures contained 5.0 μM of FDP, 1.0 ml of extract II, 20 μM of $MgCl_2$ (control contained water) in a total volume of 1.3 ml. The pH was 8.0. Incubation was at 37.5° C. At the intervals indicated, aliquots were diluted with 4 volumes of water, heated for 2 min at 100° C and centrifuged to remove the coagulated protein. Aliquots were assayed for HMP and FDP.

and 28 % F-6-P. It is evident that the expected stoichiometry of a mole of hexosemonophosphate formed per mole of FDP utilized was not obtained. This was probably caused by a further metabolism of the HMP by a series of transketolase and transaldolase reactions.

Rate of TPN Reduction by G-6-P, F-6-P and FDP: Table II presents typical rates of TPN reduction by the crude extracts when the substrate is G-6-P, F-6-P or FDP and shows the effect of Mg^{++} on the rates. It can be seen that 1) the versene treated extracts possess enzymes catalyzing reduction of TPN only with G-6-P or F-6-P as substrate, 2) the addition of Mg^{++} increases the rate of reduction with these substrates but also permits reduction by FDP. Reduction of TPN by G-6-P is catalyzed by G-6-P dehydrogenase while reduction by F-6-P and FDP is apparently caused by a conversion of these substrates to G-6-P. It is evident that the enzymes catalyzing the conversion of FDP and F-6-P to G-6-P are more affected by Mg^{++} than G-6-P dehydrogenase.

TABLE II

RATE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE REDUCTION BY GLUCOSE-6-PHOSPHATE, FRUCTOSE-6-PHOSPHATE AND FRUCTOSE-1,6-DIPHOSPHATE

PHOSPHATES	Mg^{++}	NO ADDITION
G-6-P	0.019	0.008
F-6-P	0.015	0.003
FDP	0.006	0.000

The figures represent the change in optical density at 340 mμ from 0.5 to 1.0 min in a test system given in the methods section. The extracts are type I.

Aldolase: This enzyme was measured by the hydrazine fixing technique in which the triose phosphate, formed by the splitting of FDP, reacts with hydrazine to yield triose phosphate hydrazones thereby allowing the reaction to go to completion. On incubation of 50 μM of FDP with the crude extract for 30 minutes, 14.4 μM of alkali-hydrolyzable phosphate was formed indicating the presence of aldolase. On further analysis, the triose phosphate fraction was found to be 60 % glyceraldehyde-3-phosphate (G-3-P) and 40 % dihydroxyacetone phosphate. Similar data with pea root extracts have been reported by Stumpf (25).

Glyceraldehyde-3-Phosphate (G-3-P) Dehydrogenase: As seen in figure 2, the extracts contain a DPN-linked G-3-P dehydrogenase. TPN is inactive in this system. The reaction does not proceed in the absence of arsenate. Also 1×10^{-3} M iodoacetamide completely inhibits the enzyme. Aged enzyme preparations can be reactivated by cysteine. These properties suggest that the root possesses a typical G-3-P dehydrogenase as was also found in pea seed (22). The reoxidization of DPNH on addition of acetalde-

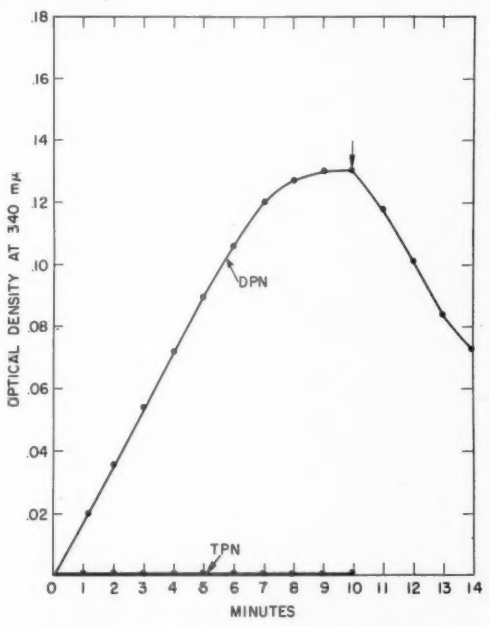

FIG. 2. Presence of triosephosphate dehydrogenase. The absorption cell contained veronal buffer, pH 8.0, 52 μM of Na arsenate, 60 μM of NaF, 12 μM of cysteine, 0.05 ml of extract I, 0.1 μM of DPN or 0.095 μM of TPN in a total volume of 3.0 ml. The reaction was begun by addition of 1.5 μM of DL-G-3-P. At arrow, 50 μM of acetaldehyde added. Readings were made against a blank containing all components except G-3-P.

hyde indicates that roots of this age still possess an alcohol dehydrogenase.

Effect of Versene on G-3-P Dehydrogenase: Inhibition of G-3-P dehydrogenase by iodoacetamide and reactivation by cysteine suggests that the enzyme depends on sulfhydryl groups for activity. Since root tissue tends to accumulate heavy metals which inhibit sulfhydryl enzymes, it was of interest to determine the effect of versene, an agent capable of removing such metals, on the activity of the enzyme during the preparation of the crude extracts. Figure 3, curve 1, indicates that the enzyme is inactive when the tissue is ground in the absence of versene but partially active when the chelating agent is present. However, as indicated in both curves, cysteine is necessary to restore full activity.

Phosphatase Activity: While animal phosphatases tend to be dependent on an alkaline pH for activity, plant phosphatases require an acid pH for activity. The data in table III agree with this general statement since inorganic phosphate was liberated from G-6-P only at an acid pH. The appearance of phosphate was inhibited by fluoride but unaffected by the addition of Mg^{++}. This phosphatase activity is not specific for G-6-P since F-6-P was also dephosphorylated. Since the conversion of FDP to F-6-P occurred at pH 8 and was sensitive to Mg^{++}, it appears that this hexosediphosphatase is not the same enzyme as that liberating phosphate from G-6-P or F-6-P. The enzyme catalyzing the breakdown of FDP to F-6-P is probably similar to that isolated from liver and designated as Gomori's phosphatase (15).

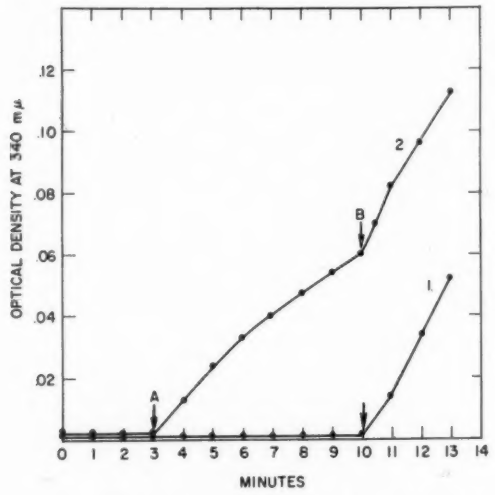

FIG. 3. Effect of versene and cysteine on triosephosphate dehydrogenase. The cuvettes contained veronal buffer, pH 7.9, 52 μM of Na arsenate, 60 μm of NaF, 0.2 μM of DPN, 0.03 ml of crystalline rabbit muscle aldolase and 0.2 ml of extract in a total volume of 3.0 ml. At arrow A, 5 μM of FDP added. At arrow B, 12 μM of cysteine added. Readings were made against a blank containing all components except DPN.

The extract used in curve 2 was prepared by grinding 9.0 gm of root tissue with 5 ml of 0.1 M $KHCO_3$ and 2 ml of versene (30 mg/ml), pH 8.0. The extract used in curve 1 was prepared without versene.

TABLE III

EFFECT OF pH ON PHOSPHATASE ACTIVITY IN PEA ROOT

MEASUREMENTS	ACID MEDIUM			ALKALINE MEDIUM		
	CONTROL	F^-	Mg^{++}	CONTROL	F^-	Mg^{++}
Final, μM PO_4	5.64	3.91	5.30	3.69	3.64	3.56
Initial, μM PO_4	3.43	3.60	3.58	3.41	3.32	3.11
Δ	2.11	0.31	1.72	0.28	0.32	0.45
pH	6.1	5.9	6.1	8.0	8.1	8.0

The test system contained 1.0 ml of extract II and 10 μM of G-6-P in a total volume of 1.2 ml. Where indicated the test system contained 20 μM of KF or 20 μM of $MgCl_2$. The mixture was adjusted to the indicated pH with 2 *N* acetic acid or 2 *N* NaOH. The incubation temperature was 30° C. At zero time and 1 hr, aliquots were diluted with 2.5 volumes of water, heated for 2 min at 100° C and centrifuged. The supernatant solution was analyzed for phosphate.

CONCLUSIONS

The present study taken together with others from this laboratory (12, 13) establishes the existence in the pea root of pathways which have been designated as direct oxidation and Embden-Meyerhof-Parnas. As in yeast, animal and other plant tissue, the dehydrogenases of the direct oxidation pathway are TPN-specific and are not inhibited by iodoacetamide. As in yeast and animal tissue, the one oxidative enzyme of the Embden-Meyerhof-Parnas scheme, triose phosphate dehydrogenase, is DPN dependent and is inhibited by iodoacetamide.

The only difference between the root and leaf with respect to breakdown of carbohydrate appears to be in the properties of triose phosphate dehydrogenase. While the root and leaf both possess a DPN-linked enzyme, the leaf tissue has, in addition, a triose phosphate oxidizing system dependent on TPN. It was noted that the absence of arsenate inhibits DPN reduction in leaf as well as root preparations. However, conclusions concerning the oxidizing enzyme(s) dependent on TPN differ. Some workers (2, 6) have reported that in crude preparations the rate of TPN reduction is unaffected by arsenate while others (1, 11) have noted an acceleration of nucleotide reduction in the presence of arsenate with partially purified preparations. These observations have given rise to the notion of a triose phosphate oxidizing enzyme unlike the classical enzyme. Recently Arnon (1) submitted evidence for such an enzyme, TPN-linked enzyme, not dependent on arsenate or phosphate plus an acceptor for activity. The enzyme(s) responsible for triose phosphate oxidation in the green leaf are under investigation in this laboratory and will be treated in a future publication.

Summary

1. Evidence is presented indicating that cell-free extracts of 12 to 15 day pea roots possess the following enzymes: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphohexose isomerase, hexosediphosphatase (fructose-1,6-diphosphate phosphatase), aldolase, triose phosphate dehydrogenase, alcohol dehydrogenase, and an acid phosphatase.

2. The glucose-6-phosphate and 6-phosphogluconate dehydrogenases are triphosphopyridine nucleotide specific and not inhibited by 1×10^{-2} M iodoacetamide.

3. The triose phosphate dehydrogenase is diphosphopyridine nucleotide dependent. The enzyme is inhibited by iodoacetamide and heavy metals and activated by cysteine. Triphosphopyridine nucleotide will not substitute for diphosphopyridine nucleotide. The absence of arsenate in the test system completely inhibits nucleotide reduction.

4. The enzyme in these extracts which converts fructose-1,6-diphosphate to fructose-6-phosphate and inorganic phosphate appears to be a specific phosphatase since it is active only at alkaline pH and is sensitive to magnesium. This is in contrast to a nonspecific phosphatase (glucose-6-phosphate and fructose-6-phosphate as substrate) which is active only at acid pH and is not affected by magnesium.

5. It is concluded that the pea root contains the direct oxidation pathway and at least the initial steps of the Embden-Meyerhof-Parnas scheme. The individual enzymes of the pea root appear to have the same characteristics as those of the green leaf with one exception; the root triose phosphate dehydrogenase is diphosphopyridine nucleotide specific.

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PHOTOREVERSIBILITY OF LEAF AND HYPOCOTYL ELONGATION OF DARK GROWN RED KIDNEY BEAN SEEDLINGS[1,2]

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Many of the light-controlled responses of plants are known to be most effectively regulated by radiation in the red region of the spectrum. Among these are such diverse phenomena as initiation of flowers in photoperiodically sensitive plants (7), germination of light-sensitive seeds (3), pigmentation of the tomato fruit cuticle (9), and growth of parts of dark-grown seedlings (1, 5, 6, 8).

In 1952 Borthwick et al (3) found that far-red radiation near 7350 Å given after a treatment with red reversed the promotive action of the red on germination of light-sensitive lettuce seeds and thus left the seeds in a non-germinating condition. Far-red was also found to reverse the action of red in the control of flower initiation in short-day plants (2, 5) and pigmentation of the tomato fruit cuticle (9). More recently Liverman et al (6) showed that the red reaction inducing expansion of bean leaf discs was reversed by far-red, and Withrow (11) demonstrated the far-red reversal of the red reaction that leads to the straightening of the plumular hook of excised bean hypocotyls.

The photoreaction has been shown to be repeatedly reversible. A succession of alternate red and far-red irradiations on light-sensitive seeds (3, 4) showed that germination depended upon the quality of the energy last received. That is, when the seeds received red energy last, they germinated, but when they received far-red energy last, they did not germinate. The red action for the control of pigmentation of the tomato fruit cuticle (9), flower initiation of cocklebur (5), and straightening of the plumular hook of excised bean hypocotyls (11) has also been shown to be repeatedly reversible by far-red. Such results indicate that the actions of red and of far-red are the reversal of a single reaction.

The purpose here is to demonstrate the existence of the same photoreversible reaction in controlling elongation of leaves and hypocotyl of intact dark-grown seedlings of Red Kidney beans and to investigate some of the details of the response.

Methods and Results

Dark Red Kidney beans were treated with Arasan and planted in well-drained redwood flats filled with white quartz sand moistened with a nutrient solution. Two parallel grooves of constant depth were made with a template in the sand in all flats. Individually weighed beans, weighing 0.4 to 0.6 gm, were placed about 2 cm apart in the grooves with their long axes vertical and raphes lowermost. The beans were then carefully covered with 3 cm of sand and each flat was moistened with additional nutrient solution. Immediately after planting, all flats were moved to a completely dark room where the temperature was $27 \pm 1°$ C. The illumination necessary for adding the nutrient solution was provided by a 3-cell flashlight equipped with a blue-green filter shown by tests to have no measureable effect on the seedlings. Irradiation treatments were given on the sixth, seventh, and eighth days, at which times the untreated plants were 90, 142, and 205 mm long. The plants were harvested on the tenth day after planting.

Preliminary Observations: When beans were grown in complete darkness, they exhibited the typical aspects of extreme etiolation. After 10 days of growth the hypocotyls were very long and the epicotyls very short. The petioles of the primary leaves had failed to elongate and the leaf blades had neither expanded nor unfolded. The plumular hook, which is first evident in the hypocotyl, was almost invariably located in the first internode just below the petioles of the primary leaves. The seedlings showed no visible chlorophyll. When dark-grown seedlings were exposed briefly to radiant energy from an incandescent-filament lamp on the sixth, seventh and eighth days after planting, elongation was inhibited in the hypocotyl and was promoted in the epicotyl and leaf petioles, and the primary leaves were induced to unfold and expand. The hook was in the petioles or in the leaf blades or it had disappeared entirely. The location of the hook seemed to depend upon the energy; disappearing more rapidly as the energy was increased. Elongation of the hypocotyl varied inversely with the energy and the length of the primary leaf blades increased directly with increasing energy (table I).

Reversibility: Red energy equivalent to about 15×10^5 ergs cm^{-2} min^{-1} at 6500 Å, the region of maximum effectiveness (10), was isolated from a bank of standard cool white fluorescent lamps (1000 fc) by a filter of two layers of red cellophane. Although the red cellophane filter was capable of transmitting far-red radiation, the emittance of the fluorescent lamps in the region between 7200 and 7600 Å was too low (less than 2%) to measurably interfere with the consequences of the red action. This spectral region was very effective in promoting the germination of light-sensitive seeds (3, 10) and in inducing pigmentation of tomato fruit cuticles (9). Preliminary experiments with dark-grown beans showed that exposure to this radiation for 2 minutes on the sixth, the seventh, and the eighth days was sufficient to induce a 38% inhibition of hypocotyl elongation and to cause leaf length to be 2.5 times that of the dark control. On the tenth day after planting these beans

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[2] This paper is based upon a thesis prepared in partial fulfillment of the requirements for the Ph.D. degree at the George Washington University, Washington, D. C.

TABLE I

HYPOCOTYL AND LEAF LENGTHS OF DARK-GROWN RED KIDNEY BEAN SEEDLINGS EXPOSED 5 MIN TO VARIOUS ENERGIES FROM UNFILTERED INCANDESCENT-FILAMENT LAMPS

RELATIVE ENERGY	NO. OF SEEDLINGS	HYPOCOTYL LENGTH	LEAF LENGTH
fcm		*mm*	*mm*
500	18	225 ± 3 *	43 ± 0.6
250	19	225 ± 2	40 ± 1.0
125	19	232 ± 2	31 ± 1.0
60	20	239 ± 2	28 ± 0.8
30	22	247 ± 3	25 ± 0.6
15	23	249 ± 2	23 ± 0.4
7	22	257 ± 2	23 ± 0.6
0	40 **	297 ± 2	16 ± 0.2

* Standard error.
** Two lots of seedlings.

normally have hypocotyls about 100 mm long under greenhouse conditions and about 300 mm long in complete darkness. Exposures to red energy resulted in a hypocotyl length of about 225 mm, equivalent to an inhibition of about 75 mm of the possible 200 mm obtainable with natural light, or about 38 %.

Far-red energy, equivalent to about 2×10^5 ergs cm^{-2} min^{-1} at 7350 Å, the region of maximum effectiveness (10), was isolated from an incandescent-filament lamp by a filter composed of 2 layers of red and 2 layers of dark-blue DuPont cellophane. Subsequent tests with dark-grown seedlings showed that far-red isolated in this way from three 300-watt, incandescent-filament, reflector-flood lamps would reverse the effect of a brief red irradiation (fig 1). The far-red radiation repromoted hypocotyl elongation and reversed the red-induced potential elongation of the epicotyl, petioles and leaf blades. Far-red also reversed the red-induced potential straightening or disappearance of the plumular hook. An energy-response study of the far-red action was made by irradiating with various energies of far-red immediately following 2-min exposure to red. A saturation effect was obtained with exposures in excess of 4 min; additional two-fold increases in exposure time did not produce measurable increases in the response of the seedlings to far-red.

As in investigations with light-sensitive lettuce seeds (4), the questions arose as to whether the red and the far-red actions were also the reversal of a single reaction in the case of dark-grown seedlings. This was answered by the effects of several alternate exposures to red and far-red. Five lots of dark-grown beans were irradiated for 2 min with red and one lot was returned to darkness. The remaining 4 lots were immediately irradiated for 5 min with far-red and again one lot was returned to the dark. The 3 remaining lots then received another 2-min red irradiation and one lot was returned to the dark, and so on

FIG. 1 (*left*). Dark-grown Red Kidney bean seedlings after being kept in continuous darkness and after being given 2 min of red radiation, 2 min of red and 5 min of far-red, and 5 min of far-red only (from left to right).

FIG. 2 (*right*). Dark-grown Red Kidney bean seedlings that had received 8-min irradiation at the indicated wavelengths in Angstrom units, on the spectrograph immediately following a 2-min exposure to red radiation.

until all lots had been returned to darkness. A red irradiation immediately prior to returning the seedlings to darkness induced leaf elongation and inhibited hypocotyl elongation. If far-red was given just before the seedlings were returned to the dark, however, the immediate effect of previous irradiations was nullified and the seedlings retained the appearance of the dark controls (table II). There is a small but possibly real increase in red response with successive irradiations. Despite these increases, the possible reasons for which are not considered here, the reversing effects of far-red are fully apparent.

TIME INTERVAL BETWEEN RED AND FAR-RED: Investigations into the control of flower initiation of cocklebur showed that reversibility depended upon the time interval between the red and the far-red irradiation (5). The time required for loss of far-red repromotion of flower initiation was about 30 min. The time required for loss of far-red control of leaf and hypocotyl length of dark-grown bean seedlings was determined by irradiating seedlings with red radiation and allowing various durations of darkness to intervene before application of far-red. Red irradiations resulted in seedlings with hypocotyls about 74 mm shorter and leaves more than twice as long as the controls (fig 3). A given energy of far-red applied immediately after the red resulted in the production of hypocotyls equal in length to those of the dark controls, and in leaves only a few millimeters longer than those of the dark controls. If the far-red was applied 1 hour after the red, the effects on hypocotyl and leaf length were almost as great as when it was applied immediately. When far-red was given as late as 8 hours after the red irradiation, its effectiveness in repromoting hypocotyl elongation seemed to be about 60 % as great as when it was given immediately after the red and 65 % as great in reinhibiting the potential red promotion of leaf elongation. About half of this apparent loss, however, was due to the growth made during the 8-hr periods between the red and far-red irradiations.

THE ACTION SPECTRUM: The only complete action spectrum encompassing both the red and the far-red action is the one determined for the control of germination of light-sensitive seeds (3), but enough critical points were determined for the far-red repromotion of flower initiation of cocklebur to show that the far-red actions controlling flowering and seed germination had the same general region of maximum effectiveness (2). Spectrographic studies were, therefore, instituted to extend this information and to determine whether the red and far-red actions demonstrated by filter experiments to control development of dark-grown beans have the same general region of maximum effectiveness as those controlling the germination of light-sensitive seeds and the flower initiation of cocklebur (2).

TABLE II

HYPOCOTYL AND LEAF LENGTHS OF DARK-GROWN RED KIDNEY BEAN SEEDLINGS EXPOSED ALTERNATELY TO 2 MIN OF RED AND 5 MIN OF FAR-RED

TREATMENT	NO. OF SEEDLINGS	HYPOCOTYL LENGTH	LEAF LENGTH
		mm	*mm*
Dark control	30	310 ± 2 *	17 ± 0.8
Red	29	230 ± 3	44 ± 0.8
Red, FR **	28	284 ± 2	21 ± 0.4
Red, FR, Red	28	222 ± 2	45 ± 0.2
Red, FR, Red, FR	31	287 ± 2	21 ± 0.4
Red, FR, Red, FR, Red	23	212 ± 3	46 ± 0.3

* Standard error.
** Far-red.

The spectrographic procedures were similar to those previously described by Parker et al (8) and Borthwick et al (1). Pertinent details are summarized here for immediate reference. The energy source of the spectrograph is the crater of a direct-current, high-intensity carbon arc lamp operated at 150 amperes and 100 volts. The slit of the spectrograph, which has an effective width of 50 Å at 5000 Å, is placed in a beam of radiant flux converging from condenser lenses and at one of the conjugate foci of the front-surfaced, concave mirror used as a focusing element. Two large glass prisms, set for a minimum deviation at 5780 Å are located in the beam converging from the concave mirror. The spectrum emerging from the prisms is reflected from a front-surfaced plane mirror that is adjustable in both horizontal and vertical direction. The spectrum produced in the focal plane of the instrument is about 10 cm broad with a dispersion of 16 Å/cm at 5000 Å and 75 Å/cm at 7500 Å. The visible part of the spectrum is spread over about 1.7 meters and contains not over 0.05 % stray flux. The variation in energy with wavelength was measured with a ten-junction thermopile calibrated with a National Bureau of Standards certified lamp. The exact positions of various wavelengths were determined from the emission spectra of barium and strontium.

Experimental technique already developed (1, 8) required that the spectrographic data be transformed by means of a reference slope or energy-response curve. This slope was obtained by measuring the response to various energies at several different wavelength regions. The slope, based on results at 6500 Å, was made linear by a plot of the response to the log of the relative incident energy (fig 4). Borthwick et al (1) found that the slope of the function was independent of wavelength but that it varied with the type of response. Therefore, separate curves had to be drawn for the hypocotyl and the leaf-length responses. Semi-log plots of these responses (fig 4) show a single linear response for leaf length and two linear functions for hypocotyl length over the range of energies used. It should be noted that such two-slope curves have been found also for leaf elongation of dark-grown peas (8) and for the elongation of the second internode of dark-grown barley (1). A two-slope curve is also obtained for leaf expansion of

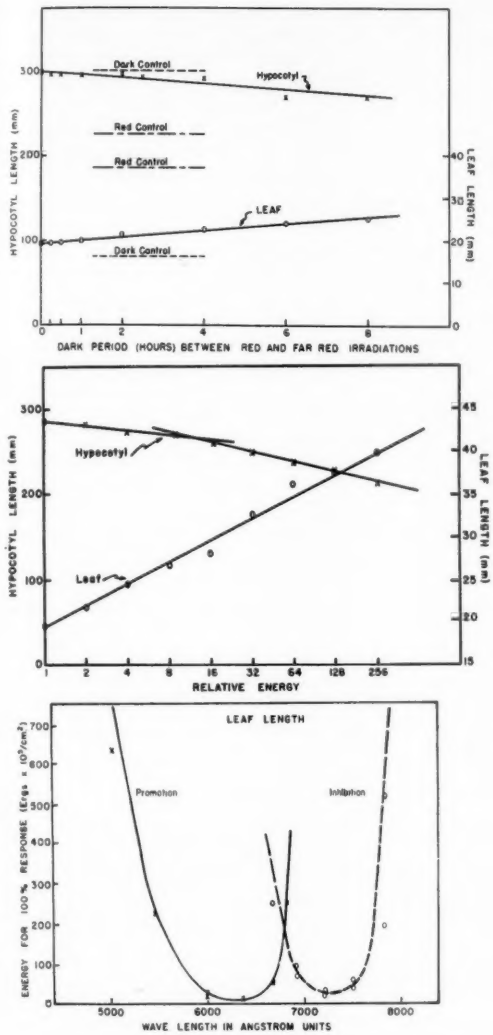

FIG. 3 (*top*). Apparent effect of various periods of darkness between red and far-red irradiations on the elongation of hypocotyl and leaf. Values for controls: hypocotyl length—dark 298 mm, red 224 mm; leaf length—dark 16 mm, red 37 mm.

FIG. 4 (*middle*). Semi-log plot of the promotion of leaf elongation and inhibition of hypocotyl elongation as a function of energy.

FIG. 5 (*bottom*). Action spectra for the promotion and reversal of promotion of leaf length of dark-grown bean seedlings.

beans when area or width is used for measurement of the response. Curves of this type are often indicative of two populations with different sensitivities to irradiation. The responses of leaf and hypocotyl to various energies of far-red result in curves of similar slope but in the opposite direction to their respective red responses.

To obtain data for construction of an energy-response curve, flats of dark-grown Red Kidney beans were placed across the spectrum from 4145 to 7100 Å and the responses to several irradiances were measured. Stations were 16.5 cm broad so that half of each flat constituted a station. The results, though preliminary, showed that the most effective region of the spectrum was in the red near 6500 Å. In order to define more precisely the portion of the red region that was most effective, the flats were divided into sections by cardboard partitions. This procedure kept the seedlings confined in a smaller spectral region and was of convenience in defining the wavelength stations. Flats of seedlings prepared in this manner were placed in the spectrum between 5830 and 7100 Å. In order to test for any promotive effect of the far-red, others were placed in the spectrum between 6497 and 8350 Å. Different lengths of hypocotyl and leaf were obtained at each station, each value resulting from a different energy. The energy required at each station to give the same length at all stations was then calculated by making use of response-log energy curve and the energy and leaf measurements for each station. The mathematical manipulations required for transformation of such data have been adequately described by Parker et al (8).

Investigations of the far-red effect were conducted along similar lines as those of the red effect. Flats of seedlings were again divided by cardboard partitions, but before they were placed across the spectrum they were irradiated with red of sufficient energy to induce more than a 100 % increase in leaf length. The flats were then placed across the spectrum between 6497 and 8350 Å (fig 2) for various periods. Leaf length values, transformed in the described manner (8), are presented as curves showing the promotive effects of radiation between 5000 and 6850 Å and the reversal effect between 6600 and 8000 Å (fig 5). On a relative energy scale the hypocotyl-elongation results would essentially be superimposed over those of leaf length. On an absolute energy scale hypocotyl and leaf length results would produce curves of similar shape with the same regions of maximum effectiveness. They would not be superimposed, however, because twice as much energy in the red region was required for the hypocotyl response as for the leaf length response. In the far-red the absolute energies necessary to produce the arbitrarily selected response limits for leaf and hypocotyl lengths were essentially the same.

DISCUSSION

Irradiation with red sets in motion a chain of reactions that, if not stopped by far-red or other means, will terminate in expression of the particular response investigated. Irradiation with far-red, if applied soon after the red, reverses the action of red in such a way that little or no measurable progress is made toward exhibition of the red response. Separation of red and far-red irradiations by various time intervals yields

evidence of the rate of decay of the products of the red treatment; that is, one can find the minimum period of time that permits the action to escape reversible control by far-red. For responses previously studied these times range from about 30 min for the complete loss of repromotion of flowering of cocklebur (5) to more than 8 hrs for a 50 % loss of regulation of cuticle color of tomato fruits (9) and germination of light-sensitive seed (4). Control of lengths of hypocotyl and leaves of dark-grown Red Kidney bean seedlings have time constants approximately the same as that for regulation of tomato cuticle color and for control of seed germination. In the light of our present knowledge the most logical explanation for the loss of far-red reversibility seems to be that a reaction subsequent to the red irradiation removes something from the system that is essential for the operation of the reversing action of far-red. If that substance were present in limited quantity and if replenishment of the supply were slow, its removal would cause a failure of reversal of the photoreaction, as illustrated by the 30-min time constant for repromotion of flower initiation in cocklebur. However, if the concentration of this essential substance were high or if it could be replenished as fast as it were removed, enough would remain available to allow at least partial control with far-red. This seems to be the case in the loss of far-red control of seed germination, tomato fruit cuticle coloration, and elongation of dark-grown Red Kidney bean seedlings.

At any rate, the close similarity of action spectra and the repeatedly photoreversible nature of the actions indicate that all of the responses tested to date are controlled by a common basic mechanism. The reaction controls flower initiation in photoperiodically sensitive plants, pigmentation of the cuticle of tomato fruits, germination of light-sensitive seed, and growth of parts of dark-grown seedlings. Since hypocotyl and leaf elongation are universally photo-controlled expressions of plant growth, it seems probable that this reversible photoreaction is of general occurrence.

Summary

1. Dark Red Kidney beans grown in complete darkness except for brief irradiations on the sixth, seventh and eighth days were harvested on the tenth day. Short exposures to radiant energy from unfiltered incandescent-filament lamps or to red radiation inhibited elongation of the hypocotyl, induced elongation of the leaves, and caused the plumular hook to straighten. When the red radiation was followed immediately by a short exposure to far-red radiation, the effects of the red were nullified and the seedlings retained the appearance of the dark controls.

2. Action of the red energy was repeatedly reversible by far-red, illustrating in part that the red and the far-red action were the reversal of a single reaction.

3. Separation of red and far-red irradiations by various time intervals gave a measure of the rate of decay of the products of the red treatment. The ability to repromote flower initiation in Xanthium with far-red was completely lost if the far-red radiation was not applied within 30 minutes after the red interruption of the inductive dark period. The apparent loss of far-red control of hypocotyl and leaf elongation of dark-grown bean seedlings was quite slow in comparison; about 40 % after an 8-hour time lapse. The real loss, however, was only about 20 % since half of the apparent loss was due to the growth that took place during the interval between the red and far-red irradiations.

4. An action spectrum was determined for both the red and the far-red action. The regions of maximum effectiveness for regulation of length of both hypocotyl and leaf were about 6400 Å for the red action and 7300 Å for the opposing far-red action. These wavelength regions of maximum effectiveness are thus essentially the same as those previously determined for the control of flower initiation and germination of light-sensitive seed.

5. Similarity of action spectra for several plant responses and the reversible features of the photoreaction are evidence of a common basic mechanism controlling these responses. The fact that such universal responses as hypocotyl and leaf elongation are controlled by this same photoreversible reaction suggests that the basic mechanism is of rather general occurrence.

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INTERACTION OF TEMPERATURE AND LIGHT IN GERMINATION OF SEEDS[1]

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Fully imbibed, viable seeds of many plants may fail to germinate. One limitation on germination may be a light requirement which can be removed by the correct poising of a reversible photoreaction (1, 2, 8). This reaction which can be repeatedly reversed by alternate exposures to red and far-red radiation also controls photoperiodic flowering responses, etiolation, bulbing, and a number of other growth responses (5). That the photocontrol is ubiquitous in all higher plants is shown by the etiolation response of seedlings (6).

The temperature, too, must be in a favorable range for seed germination which, for many kinds of seeds, is well below that best suited for subsequent growth of the seedling. A change in temperature is often effective in promoting germination. For limited periods, the temperature may greatly exceed the favorable constant range. A response to a change in temperature is also evident in vernalization, tillering, and dormancies of plant buds (9) as well as in diapause of animals. It is a phenomenon with broad significance.

Germination requirements are examined here with reference to the results of a few simple physiological experiments involving the effects of light and temperature conditions and to some extent the effects of nitrate solutions. The results suggest a pattern for the control of the germination process as well as for the growth of all organisms that must follow the temperature of the environment.

Materials and Methods

The experiments reported here were chiefly with seeds of *Lepidium virginicum* L. collected from plants grown in the greenhouse. Other seeds were obtained from various sources and all were held in dry storage at − 18° C or + 5° C. The seeds were placed in Petri dishes on two layers of standard germination blotters saturated with water or 0.2 % KNO_3 solution. The dishes were immediately covered with two or more layers of black cloth throughout all tests except when irradiations were in progress. That the cloth excluded visible light was indicated by failure of exceedingly light-sensitive seeds to respond to visible radiant energy when so protected. Durations of germination experiments varied with the kinds of seeds and with the purposes of the experiments. They ranged mainly from 2 to 5 days after the final experimental variable was introduced. Seeds with emerged radicles were counted as germinated.

Radiation sources were incandescent and standard cool white fluorescent lamps. The incandescent-filament lamp, with a filter of two layers of red and two layers of dark-blue cellophane, either with or without a supplemental water filter, was an effective source of far-red radiant energy in the region of wavelengths greater than 7000 Å for inhibiting germination. When provided with a two-layer red cellophane filter to eliminate the blue part of the visible spectrum the fluorescent lamp, because of its relatively low emission between 7000 and 8500 Å, was an adequate source of red radiant energy in the region of 5800 to 7000 Å for promotion of germination. The sources used each gave about 0.3 milliwatts/cm^2 power in the wavelength regions of maximum effectiveness. Blue cellophane filters have been found to fade when subjected to the full intensity of 300-watt flood lamps at a distance of a foot or 18 inches, but only after several hundred hours of use. Care was accordingly exercised to replace them before significant change in transmission occurred.

During germination tests in seed germination cabinets, temperatures were usually controlled within ± 1° C. Some of the experiments involved several different constant temperatures; others made use of daily temperature alternations, an 8-hour period at one temperature being followed by 16 hours at a lower temperature. In still other experiments, a single change was made from the temperature employed during imbibition to one used for a short period or during the remainder of the germination period. Exposures to radiant energy and in some cases applications of KNO_3 or other solutions were made concurrently with, before, or after the temperature change.

In all experiments with *Lepidium virginicum* L. and *Nicotiana tabacum* L. four lots of 100 seeds each were used for each treatment. Results for *Fragaria virginiana* Duchesne and *Verbascum thapsus* L. in table II were based on single lots of 100 seeds and those used for other species on two lots each. Control lots of seeds were held in darkness in all experiments and under special conditions in some experiments as required by their particular designs. All seeds of *L. virginicum* in the dark control lots almost invariably failed to germinate.

[1] Received June 15, 1955.

Conditions such as irradiation time and designs of particular experiments were established by numerous preliminary trials. The experiments reported were selected, because they have some common theme, from some 200 that have not been described. Upon repetition each gave essentially the same results in the course of several years, but the detailed results varied because the stored seeds had undergone gradual changes.

Results and Preliminary Discussion

When imbibed in water and germinated on blotters at a constant temperature of 20° C, seeds of *Lepidium virginicum* are limited to about 30 % germination as a maximum response to red radiation (table I). The 70 % of the seeds that fail to respond can, for the greater part, be made to germinate by a temperature alternation in the presence of a solution of 0.2 % KNO_3. In fact, application of either the temperature alternation or nitrate alone is largely effective. These simple observations indicate the presence of factors in addition to radiant energy which limit germination. These additional limitations are also evident in many other light-sensitive seeds, as shown by results in table II, which indicate that an alternation in temperature often enhances germination. The response to a temperature change as well as to nitrate solution is well known to workers in seed-testing laboratories.

A further distinct limitation on germination is an unfavorably high constant temperature. In fact, germination of 5 of the 9 kinds of seeds listed in table II was prevented by holding them imbibed at 30° C either after exposure to red radiation or in darkness. Germination of the other 4 kinds was reduced but not completely inhibited at 30° C. Such facts, well known to workers with seeds who use them to determine the temperatures for seed germinating chambers, are presented here merely to illustrate the essential point of a definite germination limitation. All the kinds of seeds selected were known to be light-sensitive, but they were a random sampling for temperature requirements except that all were from temperate-zone plants.

Table I

Germination of Seeds of Lepidium virginicum L. Exposed to Radiant Energy in the Red for Various Periods after Imbibition for 24 Hrs

Germination temp	Imbibition liquid	Radiation period	Germination	
			Individual lots	Average
°C		hrs	%	%
20	H_2O	0	0, 0, 0, 0	0
20	H_2O	0.25	31, 29, 18, 25	26
20	H_2O	0.50	26, 22, 31, 26	26
20	H_2O	1.0	32, 25, 32, 34	31
20	H_2O	2.0	26, 43, 28, 26	31
20	H_2O	4.0	39, 30, 39, 35	36
20	H_2O	8.0	36, 17, 29, 21	26
20	0.2 % KNO_3	1.0	93, 92, 95, 93	93
15–25	H_2O	1.0	89, 87, 86, 88	88
15–25	0.2 % KNO_3	1.0	91, 88, 92, 87	90

Table II

Germination of Water-imbibed Seeds of Various Species in Darkness and after a Saturating Exposure to Red Radiant Energy as Influenced by Constant and Daily Alternating Temperatures

Seed species	Light condition	Germination at indicated temperatures (°C)					
		15	20	25	30	35	20–30
		%	%	%	%	%	%
Brassica juncea (L.) Coss.	Red	90	48	18	2	..	40
hort. var. Giant Southern Curled	Dark	53	20	6	0	..	34
hort. var. Chinese Broadleaf	Red	58	46	47	31	..	58
	Dark	25	30	30	20	..	58
Fragaria virginiana Duchesne	Red	16	17	70	4	..	*
	Dark	2	1	0	0	..	*
Lepidium campestre (L.) R. Br.	Red	15	12	4	1	0	29
	Dark	1	1	1	0	0	1
L. virginicum L.	Red	21	32	33	0	0	73
	Dark	0	0	0	0	0	0
Nicotiana Tabacum L.	Red	94	96	94	84	8	97
hort. var. Maryland Mammoth	Dark	2	5	6	<1	0	97
hort. var. Kentucky 16	Red	87	90	92	86	35	93
	Dark	8	27	20	4	0	86
Sisymbrium officinale (L.) Scop.	Red	15	27	40	26	5	53
	Dark	1	<1	9	20	1	18
Verbascum Thapsus L.	Red	0	18	45	16	..	65
	Dark	0	0	0	0	..	7

* At 15 to 25° C germination: red—87 %, dark—7 %.

TABLE III

GERMINATION OF SEEDS OF LEPIDIUM VIRGINICUM WITH A SINGLE CHANGE IN TEMPERATURE FROM 15 TO 25° C AT THE INDICATED NUMBER OF HRS BEFORE OR AFTER A SATURATING AMOUNT OF RED RADIANT ENERGY

PERIOD BETWEEN TEMP CHANGE AND IRRADIATION	GERMINATION WHEN TEMP CHANGE OCCURS *	
	BEFORE IRRADIATION	AFTER IRRADIATION
hrs	*%*	*%*
0	87	87
4	70	89
8	65	93
12	60	86
16	71	90
20	72	82

Seeds imbibed in water for 24 or 28 hrs at 15° C prior to temp change.

* Germination 42 % at constant temperature of 20° C.

The optimum constant temperatures for germination, while not determined precisely, were in the region of 15° to 25° C for all the seeds listed in table II, with 5 of the 9 kinds being in the narrow region of 20° to 25° C. This optimum temperature range for germination is well below that for optimum growth of many plants and the limiting temperature for germination (near 30° C) is below the limiting temperature for growth.

Two features of the enhanced germination response to a change in temperature are shown by results in table III. The first of these is that a single change in temperature is effective and the second is that this change may either precede or follow the exposure to radiation by as much as 20 hours. Other experiments, the details of which are not presented, show that with longer periods of imbibition this interval during which the temperature change is effective is more restricted as also is the enhancement of germination by the temperature change. The results in the last column of table II show that the enhancement is attained even when the higher temperature is above that which suppresses germination when it is held constant. In fact, an alternating temperature of 20° to 35° C after exposure to red radiant energy increased germination of seeds of *Sisymbrium officinale* to 86 %. Under these conditions, seeds of *Lepidium virginicum* and *L. campestre* gave 36 and 14 % germination, respectively, about the same as the values at the optimum constant temperature.

Changes effected by holding imbibed seeds at temperatures as high as 35° C can be both drastic and rapid as illustrated by results in table IV. Seeds of *Lepidium virginicum* placed for 2 hours in a chamber at 35° C after imbibition at 20° C for 24 hours germinated 99 % in response to a saturating exposure to red radiant energy. The 2 hours included the interval over which temperature equilibration was being attained so that the actual time at 35° C was considerably shorter. Seeds held for 2 hours at 35° C require about one-eighth the energy for 50 % promotion of germination if the period at 35° C precedes rather than follows exposure to red radiation. The sensitivity of seeds held at 35° C either before or after radiation exposure, however, is much greater than that of seeds held constantly at 20° C. A change of this type had previously been noted by Siegel (7) for seed of *Digitaria sanguinalis* (L.) Scop. heated to 75° C for 60 minutes while unimbibed.

The percentage germination and the sensitivity to red radiant energy of *Lepidium virginicum* seeds held at 35° C for 24 hours depend on whether they receive an essentially saturating irradiation near the beginning of the 24-hour period of high temperature. Total germination and sensitivity both were lower if the seeds did not receive the initial exposure to red radiant energy (table V). The seeds given red before the period at 35° C were also given an essentially saturating amount of far-red immediately after the period at 35° C to convert the pigment to the red-absorbing form to permit measuring sensitivities to red and then far-red radiant energy.

Respiration is an essential process in germination, for seeds do not germinate in absence of oxygen. The oxygen consumptions of non-germinating seeds of *Lepidium virginicum* in darkness in either water or potassium nitrate solution at 20° C are shown in figure 1. Measurements were not made until 2½ hours after the seeds were placed in the flasks since the process of

TABLE IV

GERMINATION OF SEEDS OF LEPIDIUM VIRGINICUM IRRADIATED BEFORE OR AFTER BEING HELD AT 35° C * FOR 2 HRS

DURATIONS OF IRRADIATION		GERMINATION OF SEEDS	
RED	FAR-RED	BEFORE 2-HR PERIOD AT 35° C	AFTER 2-HR PERIOD AT 35° C
sec	*sec*	*%*	*%*
0	0	3	3
5	0	..	32
10	0	..	56
20	0	26	74
40	0	34	90
80	0	52	95
160	0	73	98
320	0	96	..
10 × 60	0	95	..
16 × 60	0	..	99
64 × 60	0	98	..
Saturation **	5	99	99
"	10	97	98
"	20	84	91
"	40	39	46
"	80	8	14
"	160	5	14
"	16 × 60	2	7

Seeds imbibed in water at 20° C for 24 hrs before placing at 35° C; returned to 20° C after treatments.

* Germinations 0, 27 and 55 %, respectively, for seeds held continuously at 20° C in the dark or with prior 4 or 64 min of red.

** Time 64 and 16 min, respectively, for lots exposed to irradiation before and after 2-hr period at 35° C.

TABLE V

SENSITIVITY OF SEEDS OF LEPIDIUM VIRGINICUM TO RED AND FAR-RED IRRADIATION AFTER BEING HELD FULLY IMBIBED AT 35° C FOR 24 HRS WITH AND WITHOUT PRIOR EXPOSURE TO RED

DURATIONS OF IRRADIATION AFTER 24 HRS AT 35° C		GERMINATION OF SEEDS	
		SATURATED WITH RED AT BEGINNING OF 35° C TREATMENT *	NOT SATURATED WITH RED AT BEGINNING OF 35° C TREATMENT
RED	FAR-RED		
sec	*sec*	*%*	*%*
0	0	5	0
20	0	34	..
30	0	..	4
40	0	42	..
60	0	..	8
80	0	58	..
120	0	..	14
160	0	68	..
240	0	..	25
320	0	78	..
480	0	..	24
64 × 60	0	87	59
64 × 60	10	66	33
64 × 60	20	49	25
64 × 60	40	31	11
64 × 60	80	20	1
64 × 60	16 × 60	24	0

Seeds held 4 days at 20° C in dark after final irradiation.

* Given 16 min of far-red at end of 35° C treatment before irradiations shown in columns 1 and 2.

imbibition occupied part of this period. The rate of oxygen consumption was not essentially influenced by the presence of nitrate. The rate for the seeds held in darkness was constant for the first 23 hours and then changed to a lower constant rate, which indicates a steady state condition for respiration of the non-germinating seeds. These results are closely similar to those previously reported for lettuce seed (4). The results with lettuce seed show in addition that respiration increases as germination starts. The enhanced respiration is the earliest physiological expression of germination that has been found (3). Its onset, in fact, takes place during a period in which germination can still be stopped by high temperature or by far-red irradiation of light-sensitive seeds.

GENERAL DISCUSSION

The ways in which the several factors mentioned in the previous section may be involved in the onset of germination are now considered. By "onset" is meant initiation of cell enlargement that results in emergence of the root. The discussion is restricted to initiation with the hope of circumscribing a single process.

The factors are: 1) imbibition of water, 2) a reversible photoreaction, which has been treated in greater detail elsewhere (2, 7), 3) a favorable temperature, with a distinct limitation at high temperatures, and 4) any changes induced by the presence of nitrate or by alternations in temperature.

Respiration, which might be treated as a primary process for germination, is considered first. Respiration, involving the reduction of oxygen and the ultimate appearance of carbon dioxide, can be considered as producing three essential types of substances: substrate compounds for reactions supplying free energy, hydrogen-transferring intermediates and intermediates for synthesis. These three types of materials, of course, are cyclically involved in respiration itself. In viable non-germinating imbibed seeds these materials are in a steady state which might be constant for periods of many hours but which would change over longer periods as substrates for respiration change, i.e., periods of a week for seeds of *Lepidium virginicum*. If the temperature is increased the rate of oxygen consumption quickly responds and a new steady state of the essential materials is established.

Germination is connected with respiration through one or more of the three essential types of materials, and can be considered as utilization of some of these materials. In a sense, it is the coupling of a particular system into the respiration cycles. When germination starts an added demand is made for the essential materials and respiration at a constant temperature is enhanced by the tendency of the added demand to reduce the steady state level of the intermediate.

The general way in which the reversible photoreaction is effective can now be considered. The pigment in the far-red-absorbing form promotes germination by directly controlling the levels of two compounds concerned in the germination process. The pigment may act as an enzyme in a reaction

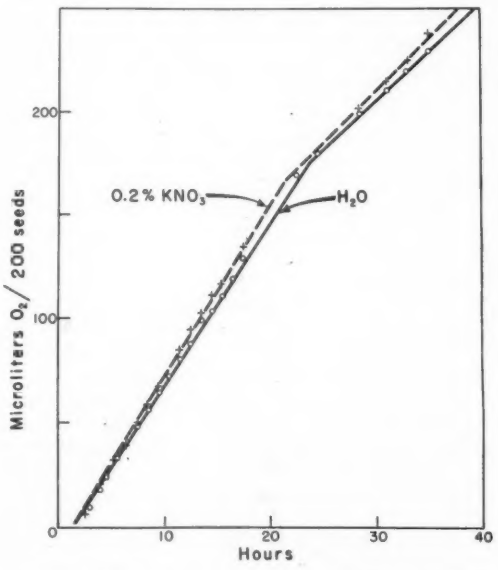

FIG. 1. Oxygen consumption at 20° C of imbibed seeds of *Lepidium virginicum* in darkness in water and in 0.2% KNO_3 solution. (Courtesy of C. E. Hagen.)

between the two compounds, may serve as an inhibitor of this reaction or may be one of the compounds. The first case, however, is the more likely and will be used as the basis for further discussion. With a favorable level of the two compounds germination will proceed in some seeds.

An important feature of the photoreaction is that, although it is present, its participation in the germination process is not necessary for all seeds. This is true not only for all of the seeds of many kinds of plants such as maize but also for some of the seeds, the dark-germinating ones, in lots that in general are light-sensitive, such as those of some varieties of lettuce. The deduction is that in such seeds the levels of the two compounds intermediated by the photoreaction are satisfactory for germination independent of the photoreaction. This implies that the levels of the compounds are controlled through other reactions. Absence of physiological evidence for photocontrol, however, does not imply that the photoreaction is absent.

Next to be considered are those imbibed light-sensitive seeds in which the pigment was placed in the far-red-absorbing form without causing germination. About 70 % of the seeds in several lots shown in table I are of this type. That the pigment is in the far-red-absorbing form in these non-germinating seeds is indicated by results such as those in table III in which a change of temperature that follows the exposure to red radiation greatly enhances germination. A change in temperature without exposure to radiation did not effect germination of *Lepidium virginicum*. Two possible reasons exist for failure of such seeds to germinate even after the necessary photoreaction: first, the necessary levels of the two compounds directly controlled by the photoreaction were not reached in these seeds, or second, there were control points in the germination process other than the photoreaction. Both are considered tenable. The first holds in some seeds that have relatively low respiration levels, either inherently as individuals of the lot or are brought to such a state by being held imbibed but non-germinating for long times. These seeds often are intractable to bring to germination.

A control of germination other than the photoreaction is best illustrated by the effect of nitrate which, as shown in table I, enhances germination after the photoreaction is removed as a limiting factor. Its value as an illustration rests upon the fact that nitrate does not influence respiration of non-germinating seeds (fig 1), which fact eliminates the confusion of a change in level of respiratory-derived materials. Nitrate does not replace the photoreaction for it has no effect on germination of seeds of *Lepidium virginicum* in darkness, nor does the photoreaction satisfy the requirement met by presence of nitrate. At least two controls, then, are present and they must enter at different places in the germination process. The process in which nitrate participates directly influences the amount of three compounds: one the nitrate itself and the others, the reacting compound and its product with nitrate. Involvement of this process in the course of germination follows from this nitrate reaction.

The contrasting effects on germination of light-sensitive seeds resulting from either holding them constantly at a temperature such as 30° C or holding them for 8 to 12 hours at this temperature before returning them to, say, 15° or 20° C, might now be approached. Results of the two conditions are strikingly different in that germination is prevented by the first and enhanced by the second. The high temperature increases the rate of oxygen consumption; so it must change the steady state levels of the three types of materials furnished by respiration. But the change in temperature also influences the rates of all the component reactions in the germination process. Temperature change very quickly affects rates of the various reactions and more slowly alters the amounts of reactants and products toward a new steady state. If the high temperature is held for a time and then lowered, a new balance of reactants for germination is afforded at the lower temperature. The most general conclusion is that this new balance of reactants, while unfavorable for germination at the high temperature, is favorable at the reaction rates involved at the lower temperature. The germination process, whatever its nature, would appear to require a delicate balance among its component reactions if for no other reason than that it can be controlled by so many factors.

Temperature changes made after the pigment is changed to the far-red-absorbing form not only influence the number of seeds that can be induced to germinate, but also, as shown by the results in tables IV and V, influence the sensitivities of the seeds to red and far-red radiation. The change alters the two sensitivities in opposite directions, a finding consistent with the participation of a reactant other than the pigment in the photoreaction. This is an indication that at a point in the germination process other than that of its coupling to respiration, the whole balance of reactions is influenced by the change in temperature. The altered sensitivities in the photoreaction, for example, could occur if the reactant other than the pigment were a hydrogen donor or acceptor involved in other hydrogen-transferring reactions either in respiration or in the germination process.

While temperature change and presence of nitrate are equally effective in causing some seeds to respond to light, they need not be equivalent in the mode of action. Nitrate probably controls more specifically through a single reaction than does temperature. Light is obligatory for the germination of each seed of *Lepidium virginicum*, but some of the seeds have a further requirement. A temperature change and the presence of nitrate are two alternative means for initiating further necessary adjustment among component reactions of germination. The conditions could be different in other seeds, such, for instance, as tobacco (table II). Some of the tobacco seeds that could be induced to germinate by exposure to light

germinated in darkness following temperature alternation; for them light was not obligatory if the temperature was satisfactory.

Summary

Effects on germination of light and of holding at several temperatures were measured on seeds of *Lepidium virginicum, L. campestre, Sisymbrium officinale, Fragaria virginiana, Verbascum thapsus, Nicotiana tabacum* vars. Maryland Mammoth and Kentucky 16, and *Brassica juncea* vars. Chinese Broadleaf and Giant Southern Curled. Germination of each seed species is enhanced by exposure to red radiation and by a diurnal alternation of temperature. A constant temperature in excess of 30° C is very unfavorable for germination.

Seeds of *Lepidium virginicum* imbibed in water and exposed to a high red irradiance for a period adequate for maximum displacement of the photoreaction gave about 30 % germination. This value was increased to 88 % or more by a temperature change from 15° to 25° C or by imbibing in 0.2 % KNO_3 solution. The effective temperature change could precede or follow the exposure to light by as much as 20 hours. Holding imbibed seeds at 35° C for 2 hours enhanced germination and affected the sensitivity of the photoreaction to radiation.

The germination process, which depends upon respiration, is controlled at different points by the several factors. The photoreaction, while possibly present in all seeds, is not obligatory for germination of all seeds. It controls the levels of two compounds which are also under control by other reactions subject to influence by temperature.

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CULTURE STUDIES ON SPHAEROCARPOS. III. THE UTILIZATION OF NITROGEN BY SPHAEROCARPOS TEXANUS[1,2]

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The literature dealing with nitrogen utilization by plants (1, 7, 8) indicates that nitrate is the principal form of nitrogen taken from the soil by most species of higher plants. Under customary field conditions, ammonium and amino nitrogen may not be available because they are converted into nitrate nitrogen by soil microorganisms. Nitrites, although they may be utilized by some plants, are rarely if ever an important source of nitrogen for plants in nature. There is evidence that some plants can absorb and utilize amino acids and other nitrogenous compounds.

Nightingale (8), reviewing utilization of ammonium and nitrate by higher plants, noted in summary: "If a liberal supply of oxygen is available in the nutrient substrate, and of cations, including particularly potassium, and if there is adequate absorption of other essential elements, then ammonium is more rapidly utilized by plants than nitrate." However, in none of the experiments cited were ammonium and nitrate simultaneously available to the plants. On the other hand, it has been demonstrated (9, 10) that when the unicellular green alga *Chlorella vulgaris* is grown in a medium containing ammonium nitrate, it preferentially absorbs ammoniacal nitrogen.

In contrast to the numerous papers on nutrition of algae, there are relatively few studies on nutrient

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[4] Died March 2, 1955.

requirements among the Hepaticae and the literature does not afford definitive evidence on the form of the nitrogen utilized by these plants. A review of the nutrient solutions used by various workers (4) shows that ammonium nitrate and various other nitrates and ammonium salts have been used. In no instance is there a discussion of the reasons for the selection of a particular salt, nor has there been any attempt to vary the form of nitrogen in order to observe the effects on the growth and development of the plants in question. In an investigation of the growth of the leafy liverwort *Cephaloziella byssacea* in "nitrogen-free" media, Griggs and Ready (6) and Griggs (5) concluded that this plant could subsist on nitrogenous impurities so small in amount as to lie below the threshold of the chemical tests available for detecting nitrates, nitrites, and ammonia, and that there was no evidence of nitrogen fixation by these plants.

Liverworts show considerable advance over Chlorella and other green algae in organization, in complexity of structure, and in specialization, particularly in the formation of multicellular sex organs and sporophytes. However, the Hepaticae are extremely simple in contrast to the high degree of organization of specialized structures among the higher plants. In order to gain some information on the form of nitrogen preferentially used by a member of the Hepaticae, a simple thalloid hepatic, *Sphaerocarpos texanus*, was studied using heavy nitrogen as a tracer and the mass spectrometer as the instrument for assay.

Materials and Methods

Two experiments were set up. In the first, pure cultures of *Sphaerocarpos texanus* Austin (including *S. europaeus* Lorbeer) were grown in liquid medium consisting of 0.200 gm NH_4NO_3 (enriched with N^{15} in the ammonium radical), 0.100 gm $CaCl_2$, 0.100 gm KH_2PO_4, 0.100 gm $MgSO_4 \cdot 7\,H_2O$, 4.0 mg $FeCl_3 \cdot 6\,H_2O$, 5.000 gm dextrose, distilled water to make one liter. The NH_4NO_3, from Eastman Kodak Company, contained 63.0 atom % N^{15} in the ammonium radical. In the second experiment, organic nitrogen as tryptone (Difco Laboratories), 0.400 gm/l, was added to the above medium.

The inoculations were made from the same male clone used previously (2, 3); and the general culture techniques were similar to those employed in these earlier experiments, except that 500-ml Erlenmeyer flasks each containing 250 ml of medium were used in place of 125-ml flasks with 50 ml of medium. For each of the two experiments, eight flasks were sterilized; seven were inoculated, the eighth one being reserved for nitrogen analysis of the autoclaved medium. The inoculated flasks were kept in a thermostatically controlled cabinet (2) at 15.5° C with illumination (100 to 200 fc) provided by "standard warm white" fluorescent lamps operated on a 20-hour light–4-hour dark cycle.

At intervals of one to three weeks individual flasks were removed from the cabinet and the plant tissue and the medium were analyzed for N^{15}, using a Nier-type mass spectrometer and employing the techniques for the preparation of the gas samples described previously (10). Molecular masses of nitrogen were used to determine the relative abundance, defined as

$$\frac{N^{29} + 2N^{30}}{2(N^{28} + N^{29} + N^{30})} \times 100 = \text{atom \% } N^{15}.$$

The plant colony was separated from the medium, washed with distilled water, examined microscopically, dried, and weighed before the nitrogen analysis of the dried tissue was made.

Results and Discussion

The results of the first experiment, in which ammonium nitrate was the sole source of nitrogen, are given in table I. The dry weights of colonies, the atom % N^{15} in total nitrogen of the plant tissue, and the atom % N^{15} in total nitrogen of the media were obtained from the individual cultures grown for various intervals of time up to 82 days. The colony in the flask removed from the cabinet at 12 days was too small to provide enough nitrogen for a mass spectrometer analysis of the tissue. The plant colony grown for 19 days, on analysis, had 32.5 atom % N^{15} in total nitrogen. This indicates that the colony had taken in and retained practically equal proportions of ammonium nitrogen and nitrate nitrogen. This is in direct contrast to the behavior of Chlorella, which, when grown in a medium containing NH_4NO_3, used ammonium nitrogen almost exclusively for building the nitrogenous components of the cells (10).

In colonies grown for longer periods of time, the mass spectrometric data on the nitrogen (table I) show that as a colony grew larger and became older a higher proportion of its nitrogen had been obtained from the ammonium radical.

Microscopic examinations of the colonies were made at the end of 33, 47, 61, and 82 days, just before the colony was dried and assayed. Figures 2 to 5 are camera lucida drawings of portions of characteristic plants from these colonies. All of the plants were atypical to some degree.

A characteristic plant from the colony grown for

Table I

Growth and Nitrogen Analyses of Individual Cultures of Sphaerocarpos texanus Grown in Medium Containing $N^*H_4NO_3$ as the Sole Source of Nitrogen

Days of growth	Plant colony dry wt mg	Atom % N^{15} in total nitrogen	
		Plant colony	Medium
0	...	...	31.0
12	...	...	36.9
19	11.0	32.5	40.9
26	16.0	34.5	42.2
33	15.3	37.5	38.1
47	25.0	40.9	32.9
61	23.8	42.0	30.6
82	28.3	44.7	29.6

* 63.0 atom % N^{15}.

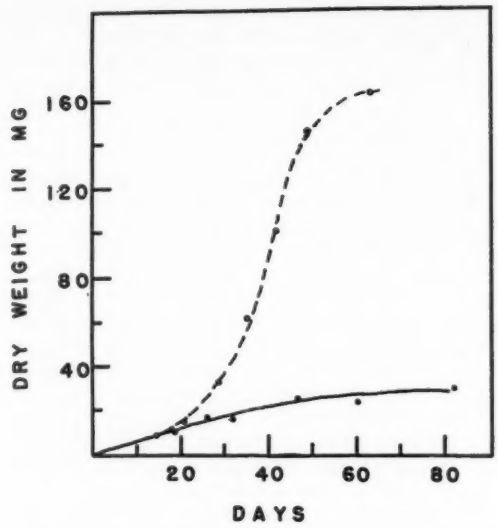


FIG. 1. Growth of *Sphaerocarpos texanus* in organic medium. ——— Growth in medium containing NH_4NO_3 as the sole source of nitrogen. - - - - Growth in medium containing NH_4NO_3 and tryptone as nitrogen sources.

33 days (fig 2) tended to be elongate rather than rosette-form and was smaller than plants considered typical for the species (fig 6). The latter had been grown in pure culture in a liquid medium containing inorganic salts and sucrose (3). The lobes (fig 2, B) were usually narrow and elongate and separated from one another by deep sinuses. The margins were usually involute. Involucres (fig 2, I) were not abundant and were usually poorly developed and deformed along one side. There were few antheridia, most of which were brown (dead). Superficial filaments and scales (fig 2, F and S) were numerous.

These same tendencies were present in the plants of all the colonies regardless of age, and they became more pronounced with increase in age of the culture. In the plant from the colony of 47 days (fig 3), the branches were elongate rather than rosette-form, the margins were deeply lobed and often several-layered from the development of large overlapping scales. Certain areas had become thick and meristematic (fig 3, M). The involucres (fig 3, I) were abundant but atypical in one way or another.

The plants from the colony of 61 days (fig 4) were similar. In certain areas of this colony the thalli produced many scales but no involucres. The plants of the colony grown for 82 days were similar. Some of them were little different from those in the younger colony (fig 2), while others were quite atypical (fig 5), in that the thalli were narrow, the wings were deeply incised in some areas, and the marginal cells were much enlarged and had divided in various ways (fig 5 at E).

The results of the second experiment, in which both NH_4NO_3 and organic nitrogen (tryptone) were available to the colonies, are given in table II. The dry weights of colonies, the atom % N^{15} in total nitrogen of the plant tissue, and the atom % N^{15} in total nitrogen of the media were obtained from the individual cultures grown for various intervals of time up to 63 days. A comparison of the dry weights of the colonies of the two experiments as given in tables I and II indicates that there has been a great difference in the growth of the colonies of the two sets. These data were used to draw the growth curves shown in figure 1. The curve for the second experiment follows the shape of a typical growth curve while that for the first experiment is extremely atypical. The extremely beneficial effect of tryptone upon the rate and amount of growth is evident from a comparison of the two curves.

The plants of the colonies were examined at the time the assays were made. In contrast to the plants grown in the nutrient containing only ammonium nitrate as the source of nitrogen, those grown in medium with tryptone added were much more robust and much more typical. The plants differed from a typical plant (fig 6) in that the growing points were usually broader and the lobes larger (fig 7). There were fewer involucres on some plants.

The addition of tryptone to the medium of the first experiment, which contained 31.0 atom % N^{15}, changed this value to 24.0 atom %. This indicates that the quantity of nitrogen contributed by 0.400 gm tryptone was slightly less than that contained in either the ammonium or the nitrate part of 0.200 gm NH_4NO_3.

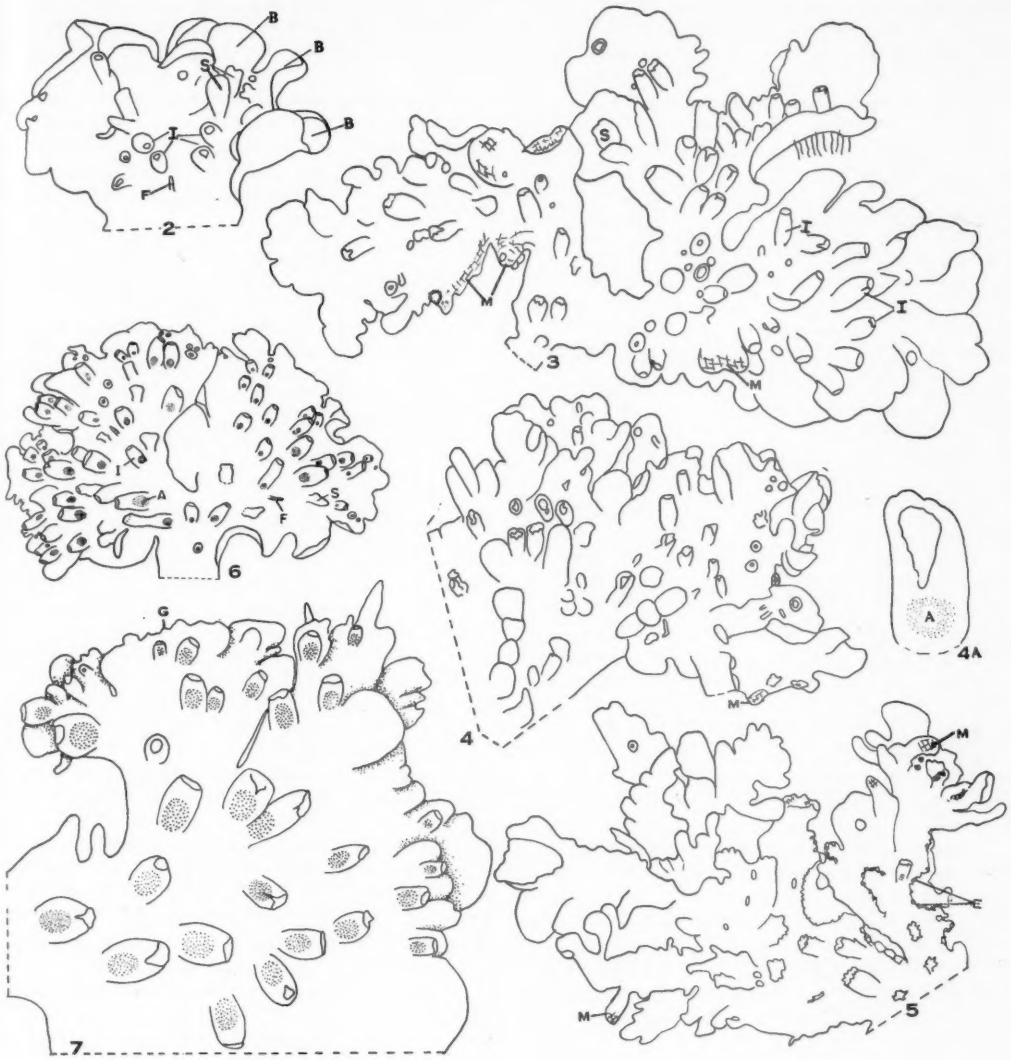
Bearing in mind that the ammonium was labeled with N^{15} and that neither the nitrate nor the organic nitrogen was so tagged, various conjectures concerning the nitrogen utilization by the liverwort in this experiment may be considered. The discussion is based on the nitrogen content of the colony of plants grown for 14 days. The value of 10.7 atom % N^{15} in this colony indicates that the plants retained proportionally more nitrate and/or organic than ammonium nitrogen. If the plants had retained equal proportions of nitrate and organic nitrogen, they would have

TABLE II

GROWTH AND NITROGEN ANALYSES OF INDIVIDUAL CULTURES OF SPHAEROCARPOS TEXANUS GROWN IN MEDIUM CONTAINING $N^*H_4NO_3$ AND TRYPTONE AS NITROGEN SOURCES

DAYS OF GROWTH	PLANT COLONY DRY WT MG	ATOM % N^{15} IN TOTAL NITROGEN	
		PLANT COLONY	MEDIUM
0	...	...	24.0
14	9.7	10.7	21.2
21	14.6	14.3	20.2
28	31.2	17.1	18.7
35	60.2	18.6	17.7
42	100.6	20.3	17.6
49	148.2	20.5	17.5
63	162.0	20.9	16.7

* 63.0 atom % N^{15}.



FIGS. 2 to 7. *Sphaerocarpos texanus*. Plants (figs 2 to 5) grown in medium containing ammonium nitrate as the sole source of nitrogen. FIG. 2. A portion of a plant from a colony grown for 33 days; B, lobe; F, filament; I, involucre; S, scale. ×16. FIG. 3. A plant from a culture grown for 47 days; I, involucre; M, meristematic area; S, scale. ×16. FIG. 4. Portion of a plant from a culture grown for 61 days; M, meristematic area. ×16. FIG. 4 A. Deformed involucre; A, antheridium. ×90. FIG. 5. A portion of a less characteristic plant of a culture grown for 82 days; E, enlarged marginal cells; M, meristematic region. ×16. FIG. 6. A portion of a characteristic rosette-form plant; A, antheridium; F, filament; I, involucre; S, scale. ×23. FIG. 7. A portion of a plant grown for 63 days in medium containing NH_4NO_3 and tryptone as nitrogen sources; G, a growing point. ×23. Figure 6 taken from Diller et al, Amer. Jour. Bot. 42: 819–829, fig. 1. 1955.

had 2.4 atoms of nitrogen from each of these two sources for every one atom originally in the ammoniacal form. Since the results of the first experiment showed that the nitrogen within the plants came in approximately equal proportions from ammonium and the less-reduced nitrate, there appears to be no basis for assuming that the addition of the tryptone would bring about so great a change that there would be a selection of nitrate nitrogen in a proportion greater than 2.4 atoms to one of ammonium or to the extent of the exclusion of organic nitrogen. The selection of nitrate nitrogen in an amount less than

2.4 atoms to one atom of ammonium nitrogen would mean that the nitrogen from tryptone would have been proportionally higher. Thus there is the strong probability that the tryptone had been an important source of the nitrogen of the plants.

Since the addition of tryptone to the medium greatly stimulated growth and was effective in producing robust, typical plants, tryptone has proved to be a valuable adjunct to the medium for culturing *Sphaerocarpos texanus*. It should be kept in mind, however, that the effect of tryptone may in part be due to the presence of additional factors in this material.

Summary

An investigation was made to determine the form of nitrogen preferentially used by the liverwort *Sphaerocarpos texanus*. There is available very little evidence in the literature on the nutrient requirements of liverworts and no evidence on the source of the nitrogen utilized when two or more types of nitrogenous compounds are simultaneously available.

Two experiments were carried out. In the first the liquid medium consisted of NH_4NO_3 (enriched with N^{15} in the ammonium radical), $CaCl_2$, KH_2PO_4, $MgSO_4$, $FeCl_3$, and dextrose. In the second experiment organic nitrogen as tryptone was an additional constituent of the medium. The plants were cultured in Erlenmeyer flasks in a cabinet with controlled temperature and light. At intervals, individual flasks were removed from the cabinet; the plant tissue was examined microscopically; dry weight of the colony was determined; and analyses of N^{15} content of the plant tissue and of the medium were made by means of a mass spectrometer.

When NH_4NO_3 was the only source of nitrogen supplied, the plants retained approximately equal proportions of ammonium and nitrate. When organic nitrogen as tryptone was available in addition to NH_4NO_3, ammonium nitrogen was used but there was evidence that the organic nitrogen was being retained in a larger proportion than the ammonium or nitrate. The question of how much nitrate nitrogen was retained by the plants could not be answered by this experiment since only the ammonium radical was labeled with N^{15} and not the nitrate or the organic nitrogen.

The addition of organic nitrogen as tryptone had a pronounced beneficial effect upon the growth and development of the colonies. The plants were very robust and much more typical and characteristic than those grown in the medium containing only NH_4NO_3 as the nitrogen source.

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BRIEF PAPERS

REDUCTION OF FERRICYANIDE BY ALGAL SUSPENSIONS[1,2]

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Light plays an indispensable role in the net reduction of carbon dioxide by photoautotrophic organisms, supplying the requisite energy for this highly endergonic reaction. However it is difficult to evaluate the part that radiant energy plays in the reduction of other substances. A number of substances are reduced by illuminated algae with a concomitant production of equivalent amounts of oxygen. Since water is therefore the ultimate hydrogen donor, it has been suggested that its photochemical dissociation is intimately associated with these reductive reactions. Fan et al (4) concluded that the observed reductions of benzaldehyde, acetaldehyde, ferricyanide, ferric salts, parabanic acid and nitro-urea in the light could be compared to the well known reduction of oxidants by isolated chloroplasts and perhaps constituted Hill reactions in vivo. Likewise Van Niel (6) considered that nitrate reduction was at least partially photochemical. However, most, if not all, of the above mentioned substances are also reduced in the dark, in which case CO_2 is produced instead of O_2. Therefore Warburg (7) and Davis (3) concluded that nitrate was probably reduced by respiratory reactions, the associated O_2 evolution in the light resulting from a secondary process, namely photosynthetic assimilation of CO_2 produced by these nonphotochemical reactions. Fraser (5) came to similar conclusions regarding the reduction of benzaldehyde and ferricyanide.

Recent research suggests that there may be some interaction between the processes of photosynthesis and respiration. It has become obvious, therefore, that these alternative hypotheses are not necessarily wholly contradictory. In an attempt to elucidate the problem the gas exchanges which accompany ferricyanide reduction have been reinvestigated. This was undertaken with the aid of a recording mass spectrometer described elsewhere (1, 2). With the mass spectrometer it was possible to follow variations in the partial pressure of both O_2 and CO_2 simultaneously and independently. Preliminary results may be summarized as follows:

1) Chlorella and Anabaena suspensions illuminated in phosphate buffer in the absence of CO_2 produced very little O_2. However, when ferricyanide was added, slow O_2 evolution ensued. The amount of O_2 produced was somewhat in excess of the amount of ferricyanide added if one mole of oxygen is taken as equivalent to four moles of ferricyanide. Figure 1 illustrates the evolution of O_2 which occurred when algae and spinach chloroplasts were illuminated in the presence of ferricyanide.

2) When ferricyanide was added to a suspension of Chlorella cells in the dark the respiratory quotient was radically altered. Oxygen uptake increased only slightly but CO_2 production was more than doubled (fig 2). The rate of production of "extra" CO_2 in the dark was approximately equal to the rate of production of O_2 by the same system in the light. This production of "extra" CO_2 required the presence of O_2; under anaerobic conditions practically no CO_2 was released by algal cells suspended in ferricyanide solutions.

3) When Chlorella suspensions were illuminated in the absence of both CO_2 and O_2 there was no detectable gaseous metabolism. The addition of ferricyanide to the illuminated system immediately initiated CO_2 production. After a short lag O_2 evolution commenced at the expense of this CO_2 production. During subsequent periods of light and dark the photosynthetic quotient was distorted in the direction of excess O_2 and the respiratory quotient in the direction of excess CO_2. This condition persisted until the total CO_2 and O_2 exceeded the amount of ferricyanide

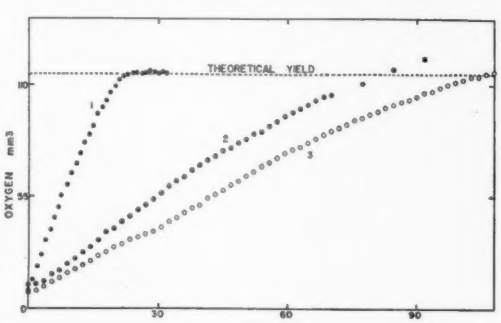

FIG. 1. Oxygen evolution in the presence of ferricyanide. *Curve 1:* Spinach chloroplasts suspended in phosphate buffer pH 6.7, 5.0 ml containing about 0.75 mg chlorophyll. *Curve 2:* Anabaena cells suspended in phosphate buffer pH 6.7, 5.0 ml containing 3.5% by volume of cells. *Curve 3:* Chlorella cells suspended in phosphate buffer pH 6.7, 3.3 ml containing 1.4% cells. Vessels flushed with He. Potassium ferricyanide solution (0.5 ml containing 20 micromoles) added from the side arm. Light intensity about 150 fc.

[1] Received May 3, 1955.

Since this paper was submitted for publication, O. Warburg and G. Krippahl have published a paper (Zeits. Naturforsch. b 10: 301–304. 1955) which confirms many of our observations.

[2] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Minnesota (NR 160-030).

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added, whereupon the quotients of both processes returned to values close to unity (fig 3).

These results imply that ferricyanide is reduced in the dark by respiratory mechanisms involving the production of CO_2, ferricyanide supplementing but not replacing O_2 as a respiratory hydrogen acceptor. The dark reduction of ferricyanide seems to differ from the reduction of O_2 in that it is, from the standpoint of the organism's economy, an energy requiring process rather than an energy contributing process. This is indicated by the facts (a) that O_2 uptake increases rather than decreases during ferricyanide reduction and (b) that ferricyanide reduction (as measured by CO_2 production) does not occur anaerobically.

The respiratory machinery is probably also involved in the light reaction. The rates of ferricyanide reduction are so similar in light and dark that the existence of a common rate limiting step seems likely. Moreover CO_2 production rather than O_2 production occurs in the light during the induction phase of photosynthesis. This observation that illuminated anaerobic cells, unlike similar cells in the dark, are immediately able to reduce ferricyanide indicates that light can replace O_2 in mediating ferricyanide reduction. Consequently it is suggested that even during the induction phase light may supply some of the products of normal aerobic respiration.

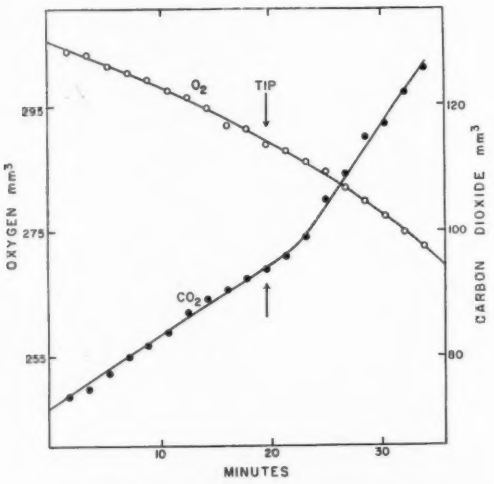

FIG. 2. Effect of ferricyanide on the dark respiration of Chlorella. Vessel contained 5.0 ml suspension of Chlorella cells (1.4 %) in phosphate buffer pH 6.7. Gas phase in vessel contained 1.1 % oxygen at the beginning of the experiment. Potassium ferricyanide solution (0.5 ml containing 20 micromoles) added from side arm at the time indicated by the arrows.

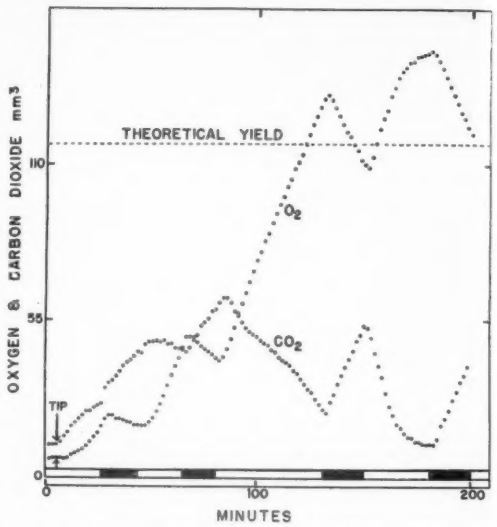

FIG. 3. Effect of ferricyanide on the gaseous metabolism of illuminated anaerobic Chlorella cells. Vessel containing 5.0 ml of a suspension of Chlorella (2.2 % cells) thoroughly flushed with He, closed and illuminated with light intensity of 150 fc for several minutes. Potassium ferricyanide solution (0.5 ml containing 20 micromoles) added from side arm at the time indicated by the arrow.

The author wishes to thank Dr. Allan Brown for his assistance in this research.

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SIMPLE LARGE SCALE ULTRAFILTRATION USING OSMOTICALLY FORCED DIALYSIS[1]

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In the preparation of protein concentrates, particularly from sources dilute with respect to protein, it is useful to employ ultrafiltration. However, in order to achieve convenient filtration rates, it has heretofore been necessary to employ high pressures and especially prepared membranes (1). However, Schneider and Wallenius (3) concentrated the protein (0.02 to 0.03 %) in 4 to 9 ml of cerebrospinal fluid to 0.1 to 0.2 ml in 24 hours by suspending the fluid in a cellulose dialysis sac in 500 ml of a 10 to 20 % solution of dextran. As the dextran is too large to pass the pores of the membrane, the water, salts and other low molecular weight substances are osmotically removed from the interior of the sac. They cite Ewerbeck (2) as independently using this principle, while Slater and Kunkel (4) substituted polyvinylpyrrolidone. We have applied this principle to large scale concentrations with a slight modification in procedure and with the substitution of the less expensive polyethylene glycol (Carbowax 6000; Carbide and Carbon Chemicals Co., 30 East 42nd St., New York 17, New York) as the high molecular reagent. Thus, with only cheap chemicals and common laboratory equipment it is possible to concentrate the protein (and other colloidal materials) from large volumes of dilute solution. We propose the name, "osmotically forced dialysis," for this method of concentration. The essentials of our procedure are as follows:

Step 1. Load the liquid into small diameter cellulose dialysis tubing of any convenient length. Lay the tubing in a container between layers of dry Carbowax 6000, using a minimum weight in grams equal to one-fourth the volume in milliliters.

Step 2. When there is no more liquid in the tubes, transfer them to distilled water for dialysis. After a time, force the liquid to one end of the tube; rinse the remainder and discard it.

Step 3. Repeat concentration as in Step 1.

Step 4. Rinse concentrate from tubes and freeze dry. If necessary, any contamination of the dry product with Carbowax probably can be removed with dry methanol.

In one run, 3800 ml (11 % dry solids) of juice from apples rotted with *Botryosphaeria ribis* Gross. and Dug. were concentrated to 8.6 gm dry solids (0.23 %), a concentration of 440 fold over the original volume, or 50 fold over the original dry weight. Of this crude product, 13 % dissolved in dry methanol. In another run, 15,340 ml were processed, yielding 29.4 gm (0.19 %). The times involved were about: Step 1—18 hours; Step 2—12 hours; Step 3—12 hours. Steps 1 and 3 can be speeded by the use of more Carbowax.

The efficiency of any manipulation of proteins demands the avoidance of denaturation. The nature of the extracts used prohibits any generalizations on this matter, but there do not seem to be any fundamental difficulties (3, 4), as proteins are generally more stable when concentrated than when dilute. If difficulty is found, it may be wise to purify the Carbowax by precipitation from an organic solvent (e.g., acetone) as it is known to contain methyl hydroquinone (absorption maximum at 290 mμ), or substitute polyvinylpyrrolidone (General Aniline and Film Corp., 435 Hudson Street, New York 14, New York). In this work all lengthy procedures were carried out in a cold room or refrigerator. Recovery of the one enzyme studied (a pectinase) was complete when the lyophilized powder was compared with the original juice. After washing with cold dry methanol and ether at room temperature, the recovery was only 75 %. The lyophilized powder was mostly polysaccharide, which may have helped prevent denaturation.

The choice of the high molecular reagent in this procedure is somewhat arbitrary. Carbowax 6000 has about the smallest feasible molecular weight (ca 6000), as Carbowax 4000 goes through cellulose tubing too easily. Besides being more economical, Carbowax 6000 is easier to handle than polyvinylpyrrolidone. The latter is a sticky powder while the former comes as brittle waxy flakes.

[1] Received June 9, 1955.

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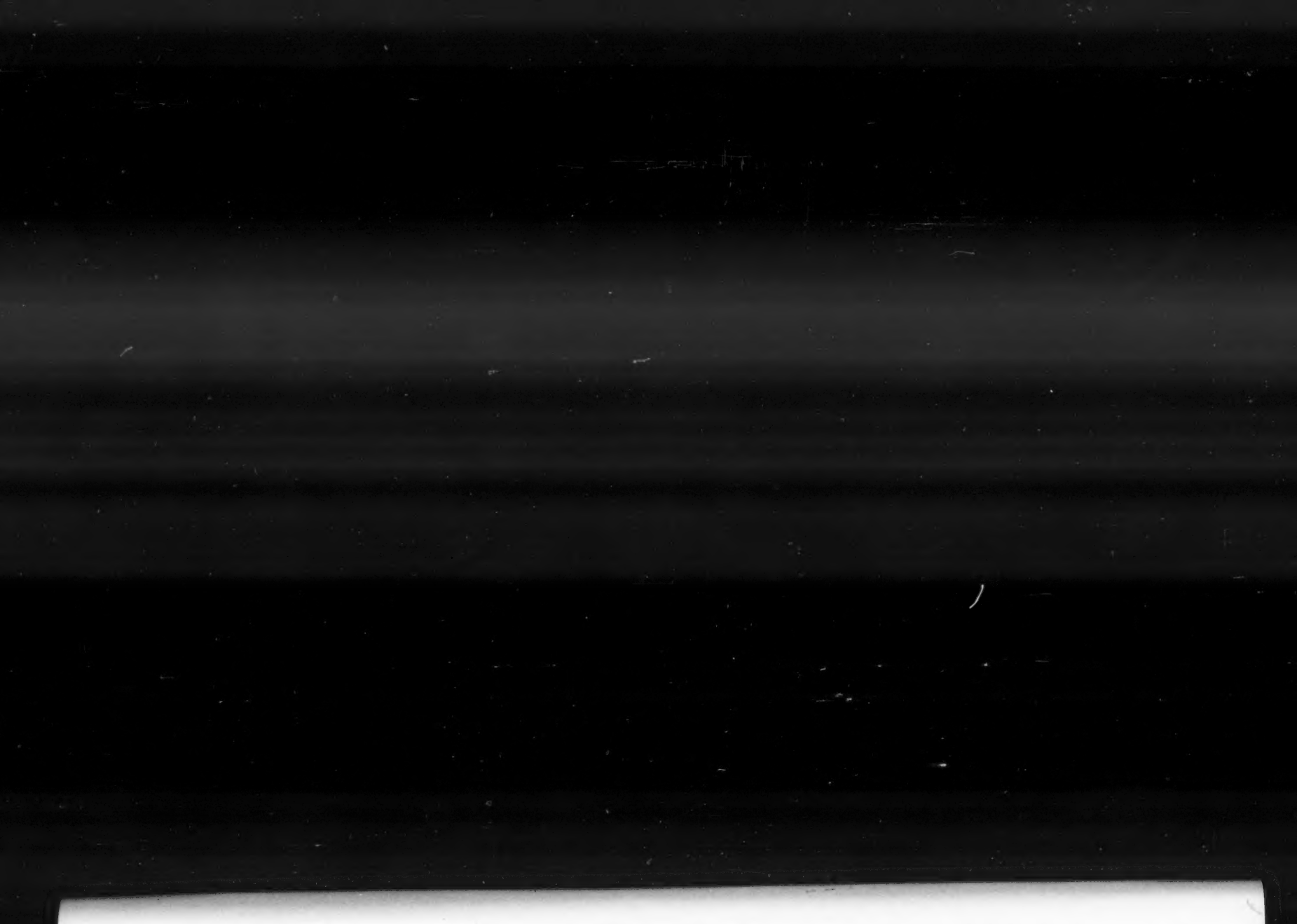
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